Inflammation requires an anti-inflammatory/regenerating protein REG3β to induce insulin resistance

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
Zeping Zhao
August 2018

INFLAMMATION REQUIRES AN ANTI-INFLAMMATORY/REGENERATING PROTEIN REG3β TO INDUCE INSULIN RESISTANCE

Zeping Zhao, Ph.D.

Cornell University, 2018

Abstract

Inflammation is a well-accepted cause of insulin resistance (IR), and anti-inflammatory treatments (AITs) are supposed to improve insulin sensitivity. Herein, we revealed that knockout of a potent anti-inflammatory protein: murine regenerating islet-derived protein 3β (REG3β), paradoxically enhanced insulin sensitivity, and completely blocked development of IR induced by lipopolysaccharide (LPS) or dextran sulfate sodium (DSS)-mediated inflammation in mice. These knockout effects were reversed by exogenous REG3β administration, and conferred by ablating two previously-unknown bindings of REG3β to transmembrane proteins exostosin-like glycosyltransferase 3 (EXTL3) that suppressed expression of insulin receptor (INSR) and protein kinase B (AKT) and cytokine receptor C-X-C motif chemokine receptor 4 (CXCR4) that inhibited phosphorylation of INSR and AKT. Prior conflicting effects of anti-inflammatory drugs on insulin sensitivity could now be explained by their different ability to alter tissue REG3β protein. Suggested human homologue regenerating islet-derived protein 3α (hREG3α) exhibited similar functions to those of REG3β. Our work unveiled REG3β as a potent negative determinant of insulin sensitivity and an essential mediator for inflammation to induce IR. These findings drastically revise current theory on the relationship of inflammation and IR, and offer an exciting new therapy target of IR and a safer, alternative prospect to treat inflammation in diabetes.

Biographical Sketch

Zeping Zhao attended high school at High School Attached to Harbin Normal University, in Harbin, Heilongjiang, China. Immediately following, he enrolled as an undergraduate Animal Science student in China Agriculture University Department of Animal Science. He graduated with his Bachelor of Agronomy degree in Animal Science in May, 2012. After graduation, he enrolled as a graduate Animal Science student in the Animal Science Department at Cornell University under the supervision of Dr. Xingen Lei, where he performed research in molecular human nutrition and biology focused on the role of Reg3β and Reg2 in inflammation or anti-inflammatory drugs induced insulin resistance or insulin secretion and how high selenium intake affected glucose, lipid and protein metabolism.

Acknowledgements

First of all, I would like to thank my family for all of their support. I was lucky enough to be born in an intellectuals' family. Even through these years, the development of China is very fast and everybody has a chance to go to university but looking back to the years when my parents grown up, only a small group of people could go to universities by their hardworking. My parents built me a relatively good study environment by their hardworking. They are the one who taught me to open my eyes to observe the world instead of just focusing on events happening in front of me. In 2002, they sent me out to Australia for traveling by myself and this trip made me get aware of the culture of another country and how the university taught in a western world country. This trip made me make my mind to studying abroad. My family is not very wealthy and different with some rich families who can give their kids whatever kinds of luxury clothes or staff, but at least my parents got all the support I needed to be a better person. They supported me to get enroll into a private middle school and go outside to exploring the other side of the world. They supported me to go to the best high school in our city even when I almost failed my enrollment exam. They supported me to pursue my interested in biology major in university. They had also supported me to pursue my dream to apply a graduate school in the US and they have never stopped to generously support me on economy when I have problems to payoff my bills in the US. They taught me to be a hardworking person with their own actions, as they would stay up to 4am to finish their job and still needed to get up at 7am to prepare breakfast for me. Without them, I wouldn't be able to reach so

far. I felt lucky enough compared with kids who was born in families that couldn't support their dream and needed them to drop their education to help their families.

Second, I would like to thank Dr. Lei for all of his supports, suggestions and advice along my six-year study in Cornell. In my second year, Dr. Lei supported me to go to San Diego to present my work in front of all the wonderful scholars in Experimental Biology conference, which gave me very precious experience in academic presentation. Afterwards, Dr. Lei continued to support me to present in Boston and Chicago in Experimental Biology conference. These experiences taught me how to be an acceptable academic presenter and how to prepare my materials and to show it to all the audience. On research, Dr. Lei also gave me many valuable guidances and suggestions. At the beginning of my Ph.D., to get me ready for the real challenging project, Dr. Lei assigned an elementary research to me to get me trained to be an independent researcher who could perform experiments by myself. This successfully helped me to transfer from an undergraduate without subjective initiative to a graduate student that could lead my own research. Later, Dr. Lei had me join Dr. Yun and Xi Yan as a team to finish the Reg2 project. This made me get a basic idea on how to perform a complete molecular gene function study. These experiences or training are all valuable in supporting my dissertation research. Dr. Lei's suggestion on my experiment design and directions of my research also offered me huge helps to allow me to perform this complete and significant research. I would also like to thank all my committee members who offered me suggestion in my proposal and my research direction when there were some impropriate

designs in my proposal or when my research hit a dead end. Without all these suggestions, I won't be able complete these projects during my Ph.D. period.

I would like to thank our previous technician, Carol. Carol was such a kind person who helped from all aspects, both in my research and in my life. Whenever I needed help with operation on mice, she would generously offer her help. She also offered many supports on western blot, co-immunoprecipitation, histology. Whenever we needed the reagents, she placed orders for us. She played an important role as the manager of this lab. Without her, many of our research wouldn't have been complete so neat and quick. She also supported me in my life when I broke up with my partner and was in a very gloomy mood, she was the one who took me to the psychologist. I am very grateful for all her caring and supporting. I would also like to thank all the lab members who offered me help before. Lvhui taught me RNA isolation. Dr. Yun taught me cell culture, siRNA, luciferase assay and ChiP assay. He also gave me an example on how to arrange my time efficiently in the lab by conducting multiple experiments at the same time. Xi Yan taught me western blot, AP-assay, insulin and glucose tolerance test, plasma lipid profile measurement, islets isolation and protein purification. Yixuan helped me develop better skills in gene cloning, protein expressions in E. Coli and yeast and protein purification. Matt helped a lot on bioinformatics method to characterize my protein. Zhixiong showed me lipid extraction. Dr. Kim offered lots of supports in the high Se and high fat feeding research. He showed me how to lead a complete animal nutrition study. Meghan taught me the fatty acid extraction and profiling skills. Other than these technical supports, including Dr. Ricardo, Dr. Tao, Dr. Debrup, Chris and Viju and together with all our lab

members mentioned above offered me a good environment to discuss science, stimulate thoughts, new findings, new ideas, and experiences on future career. I am feeling luck by having them as my lab mates to learn from and support each other during my Ph.D. period. I would also like to thank Ankur Chaudhury for preparing all the preliminary data for this dissertation research. Even though I have never met him, this study wouldn't have existed without his hard work. Also I would like to thank Dr. Sheng Zhang for supporting us in performing mass spectrometry in Cornell Proteomics Facility and all the staffs that offered me help on Co-focal microscope imaging in the Cornell Imaging Facility. I will also never forget all the staff that offered me support in Department of Animal Science especially Joanne who always help us to order materials, proofreading and many other daily supports. Even though these helps may not be seems as critical as previously mentioned but without these supports, I would have been slowed down a lot. These supports are indispensable. This project is supported by the National Institutes of Health (DK 53018).

At last, I would like thank all my friends that I made since I arrived the US so far, especially Kaleb. They supported me when I was down and feeling hopeless. They made me never missed my home. They are every part of my life in Cornell University. Kaleb had helped me to gain the courage to tell my best friend and my parent who I really was. By his support, I had a chance to experience the real American countryside life and joined the new community where I could open myself to everybody that I didn't have to hide myself. That enlightened my life. Even though, in this period, some terrible things happened, but I had never regretted. If I were given another chance, I would still choose

to get to know them. What didn't hit me down made me stronger. Even through that I lost my car and I had to help some of my friend to deal with a lawyer that I thought I would never have to deal with when I was in China, but they are part of my life that made me stronger and better in addressing these situations. So I truly appreciate all the love they offered me and I love them as well.

TABIE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION

Akt1, AKT serine/threonine kinase 1;

Akt2, AKT serine/threonine kinase 2;

Akt3, AKT serine/threonine kinase 3;

AP, alkaline phosphatase;

ß-Actin, beta-actin;

C/ebpβ, CCAAT/enhancer binding protein beta;

C/ebpd, CCAAT/enhancer binding protein delta;

Cxcl12, C-X-C motif chemokine ligand 12;

Cxcr4, C-X-C motif chemokine receptor 4;

EGFP, enhanced green fluorescent protein;

Extl3, exostosin like glycosyltransferase 3;

Gpx1, glutathione peroxidase 1;

His-tag, polyhistidine-tag;

Hnf3b, Forkhead Box A2;

Ikbkβ, inhibitor of nuclear factor kappa B kinase subunit beta;

Il1β, interleukin 1β;

Il-6, interleukin 6;

Il17, interleukin 17;

Il22, interleukin 22;

Insr, insulin receptor;

Irs1, insulin receptor subunit 1;

Irs2, insulin receptor subunit 2;

Jnk, the c-Jun NH2-terminal kinases;

KO, knockout

mCherry, mCherry fluorescent protein;

Nfkb1, nuclear factor kappa B subunit 1;

Nr3c1, Glucocorticoid receptor;

P38, p38 mitogen-activated protein kinases;

P65, RELA proto-oncogene, NF-KB subunit;

P-Akt, phosphor protein kinase B;

PBS, phosphate buffered saline

Pi3k, phosphoinositide 3-kinase;

P-Insr, phosphor insulin receptor;

P-Jnk, phosphor c-Jun NH2-terminal kinases;

P-P38, phosphor p38 mitogen-activated protein kinases;

Pten, phosphatase and tensin homolog;

Reg3ß, regenerating islet-derived *3ß*;

Socs3, suppressor of cytokine signaling 3;

Tnfα, tumor necrosis factor-alpha;

wk, week

WT, wild type

Wt1, Wilms tumor 1

CHAPTER ONE

INTRODUCTION

1.1 Current research on inflammation and insulin.

Systemic inflammation is a well-recognized contributor to the development of IR (Feve and Bastard, 2009; Finucane et al., 2015; Ghorpade et al., 2018; Pickup et al., 1997; Tian et al., 2016), whereas AITs are supposed to prevent or ameliorate the development (Ghorpade et al., 2018). Experiments confirmed the role of cytokine, tumor necrosis factor alpha (TNFα), and its receptor-associated factor (TRAF1) in the development of insulin resistance (Hotamisligil et al., 1993; Xiang et al., 2016). Same as TNFα, interleukin 1 beta (IL-1β) was demonstrated to inhibit insulin sensitivity (Finucane et al., 2015; Jager et al., 2007). Moreover, a recent study demonstrated interleukin 1 (IL-6) produced by macrophages stimulated adipose tissue inflammation leading to hepatic insulin resistance (Perry et al., 2015). Similarly, natural killer cells and conventional dendritic cells were proved a crucial link between visceral adipose tissue inflammation and insulin resistance (Macdougall et al., 2018; Wensveen et al., 2015). Additionally, research revealed that dipeptidyl peptidase-4 (DPP4) secreted from hepatocytes promoted adipose inflammation and further promoted insulin resistance. This finding emphasized the crosstalk between inflammation and hepatic insulin resistance (Ghorpade et al., 2018). However, inactivating or suppressing key cytokine and interleukin genes in the currently-recognized inflammation-induced IR pathway failed to improve or even impaired insulin sensitivity. Likely, even though overexpression of TNFα contributes to

the development of insulin resistance, knockout of TNFα failed to protect mice from insulin resistance efficiently (Ventre et al., 1997; Wascher et al., 2011). And, a recent paper even showed that down-regulated TNFα worsened glucose homeostasis (Wu et al., 2018) and from two other papers, IL-1 receptor antagonist (IL-1Ra) application showed two reversed effects on IR (Larsen et al., 2007; van Asseldonk et al., 2015). Meanwhile, anti-inflammatory drugs exert conflicting effects on alleviating the inflammation-induced IR. Sodium salicylate was discovered to improve insulin sensitivity (Ebstein, 2002) and a 2-week high-dose aspirin treatment was found to be able to bring fasting plasma glucose back to normal (Ford et al., 2015; Reid et al., 1957), though strong side effects limited its application in insulin resistant patients (Garcia Rodriguez et al., 2016). Statins as an anti-inflammatory drug was reported to have little effect on improving (Chan et al., 2002) or even impairing insulin sensitivity in a subsequent clinical study(da Silva et al., 2018; Preiss et al., 2011). Moreover, applying dexamethasone, another anti-inflammatory drug, decreased 30-40% insulin sensitivity (Buren et al., 2002; Pereira et al., 2014; Perry et al., 2003). Some drugs like Tocilizumab showed varied responses (Chen et al., 2015; Makrilakis et al., 2015; Tournadre et al., 2017). Apparently, the relationship between inflammation and IR and the underlying mechanism are far more complicated than our current understanding.

1.2 REG3β.

Murine REG3β (REG3β) is one of the 12 identified islet-derived regenerating protein family (REG) members (Iovanna and Dagorn, 2005; Liu et al., 2008). This 17-kDa,

secreted protein (Iovanna and Dagorn, 2005) is highly expressed in pancreas and intestine (Gironella et al., 2013; Gironella et al., 2005). The Clustal Alignment analysis (Thompson et al., 2002) suggests human regenerating islet-derived protein 3α (hREG3α) and murine REG3β (mREG3β) are homologues and research also demonstrated that hREG3α has similar function with REG3β (Lai et al., 2012; Mukherjee et al., 2014; Orelle et al., 1992; Pickert et al., 2009). REG3β is known as a regenerating protein since it was reported to be detected in the serum or the pancreatic juice of pancreatic ductal adenocarcinomas patients (Xie et al., 2003) and promoted pancreatic cancer growth (Folch-Puy, 2013; Gironella et al., 2013; Liu et al., 2015) and tissue regeneration in rats with acute pancreatitis (Keim et al., 1991), pancreatectomy mice (Rankin and Kushner, 2010), injured intestine regeneration (Lindemans et al., 2015) and the brain tissue of Alzheimer patients (Duplan et al., 2001). Absence of REG3β significantly impaired heart-healing processes (Lorchner et al., 2015). REG3β was also reported to play a role in antimicrobial functions (Miki et al., 2012) by inhibiting intestinal bacterial translocation upon oral infection with Gram-negative *Salmonella* (van Ampting et al., 2012). Moreover, REG3β is known as an important protein involved in the anti-inflammation of the pancreas and intestine by suppressing NF-kappa B activation (Aden et al., 2016; Folch-Puy et al., 2006; Gironella et al., 2007; Ogawa et al., 2003). However, its involvement in the inflammation-induced IR could never have been imaged until we serendipitously found exceptionally high insulin sensitivity in the REG3β$^{-/-}$ mice used for an early study (in 2013) of hepatotoxicity (Yun et al., 2013). After knowing its dual role in anti-inflammation while impairing insulin sensitivity, we hypothesized that REG3β might serve as an intriguing mediator to connect inflammation to the induced IR,

responsible for the paradoxical effects of cytokines, interleukins, and anti-inflammatory drugs on insulin sensitivity.

To understand how REG3β mediate insulin sensitivity, we explored the mechanism on transcription and phosphorylation. To explore the possible mechanism of REG3β on the transcription of insulin signal pathway genes, we performed a database search by TESS (http://www.cbil.upenn.edu/tess/) to identify transcription factors target to the proximal promoters of these genes. Among the transcription factors we found during our searching process, glucocorticoid receptor (NR3C1) become our primary target for three reasons: 1) previous research showed that NR3C1 was associated with glucose stimulated insulin secretion (GSIS) (van Raalte et al., 2012) and insulin level (van Rossum et al., 2002). Moreover, the inhibition of glucocorticoids on insulin sensitization is documented (Geer et al., 2014). Additionally, ChIP assay identified several target genes of NR3C1 including insulin signaling transactivation genes (Wang et al., 2004) [insulin receptor substrate 1 (*Irs1*)]. These findings demonstrated the involvement of NR3C1 on insulin sensitivity; 2) REG3β was reported to stimulate M2 macrophage polarization with high expression of NR3C1 indicate that REG3β might regulate NR3C1 expression (Wynn and Vannella, 2016); 3) we identified several binding sites of NR3C1 on the proximal promoter region of *Insr/Akt1/Akt2*.

Moreover, to link the endocrine-like, extracellular REG3β to the possible action of NR3C1 in the transcriptional inhibition of insulin signal pathway genes needs a transmembrane protein as a binding partner of REG3β. We conducted an exhaustive literature search and found EXTL3 (a membrane protein and a glycosyltransferase that

regulates the biosynthesis of heparan sulfate) (Busse et al., 2007) as the most promising candidate. This was because: 1) EXTL3 is be able to bind the human homologue of REG3β: hREG3α and regenerating islets derived protein 1 alpha (REG1α) that all contain C-type lectin domain, which is reported to bind with EXTL3 (Van Ba et al., 2012; Wu et al., 2016b); and 2) heparan sulfate proteins, produced by the catalysis of EXTL3 (Busse et al., 2007), could activate NR3C1 via stepwise interactions with fibroblast growth-factor and fibroblast growth-factor receptor complex to activate mitogen-activated protein kinase (ERK1/2) (Galanternik et al., 2015), the complex of tau 1 transactivation domain of NR3C1(Dahlmanwright et al., 1994) with cAMP response element binding protein (Almlof et al., 1998). Thus, REG3β has a good potential to bind to EXTL3 and further activate NR3C1 to inhibit the transcription of insulin signal pathway genes.

To explain the possible intriguing mediator role of REG3β to connect inflammation to the induced IR, we need to explore the possible mechanism of REG3β on the phosphorylation inhibition of insulin signal pathway proteins under inflammation. We conducted an exhaustive literature search and identified suppressor of cytokine signaling 3 (SOCS3) (an anti-inflammatory protein) as the primary candidate for the following reason: 1) both REG3β and SOCS3 can be stimulated by either inflammatory or anti-inflammatory cytokines like TNFα and IL-6 (Zhang et al., 2006; Zhang et al., 2014). Also REG3β was reported to activate signal transducer and activator of transcription 3 (STAT3) (Lorchner et al., 2015), which is a stimulator of SOCS3 (Ramadoss et al., 2009). This made REG3β a very promising regulator of SOCS3 as well; 2) SOCS3 was

proved as an important insulin sensitivity suppressor under inflammation condition by inhibiting the phosphorylation of INSR and AKT (Carow and Rottenberg, 2014); 3) to our best knowledge, other inflammation induced insulin sensitivity suppressors like p38 mitogen-activated protein kinases (P38) was not reportedly correlated with REG3β.

Also, to link extracellular REG3β to the possible action of SOCS3 in the phosphorylation inhibition of insulin signal proteins needs a transmembrane protein as a binding partner of REG3β. However, after an exhaustive literature search, EXTL3 was not found to have any association with the phosphorylation of insulin signal proteins. But, we identified CXCR4 and glycoprotein 130 (GP130) (well-known transmembrane inflammatory cytokine receptors (Wescott et al., 2016) that could be stimulated by inflammation) as our primary candidates for exerting the function of REG3β on phosphorylation inhibition of insulin signal proteins for the following reasons: 1) SOCS3 was shown to be directly stimulated by CXCR4 and GP130 (Takekoshi et al., 2013) in the presence of its ligand C-X-C motif chemokine ligand 12 (CXCL12) or IL-6; 2) the CXCL12/CXCR4 complex or IL-6/GP130 complex was speculated to be the target of REG3β in cancer cells (Loncle et al., 2015); and 3) other important cytokine receptors were not reported as targets of REG3β. Thus, we hypothesized that REG3β is likely binding to CXCR4/GP130 and further activates SOCS3 to inhibit phosphorylation of the insulin signal pathway proteins.

CHAPTER TWO

OBJECTIVES AND EXPERIMENTAL PROTOCOLS

2.1 Aims

Regenerating islet-derived protein 3β (REG3β) has been identified as an anti-inflammatory protein essential in tissue regeneration and cancerogenesis. Since the reason for inconsistent effects of anti-inflammatory drugs on treating insulin resistance is still unknown, our study was designed to test 1) if REG3β acted as a negative determinant of the baseline insulin sensitivity; 2) if REG3β was the key mediator for the systemic inflammation induced IR and 3) what the subsequent mechanism of REG3β on insulin signaling was.

2.2 Experimental Procedures

2.2.1 Animals, treatments, and metabolic response detection.

The genetic background of REG3β knockout (KO) and wildtype (WT) mice (129/S6×C57BL/6), feeding and housing conditions, and tissue sampling were the same as previously described (Wang et al., 2008). All mice used in this research were male and 2-months old unless otherwise indicated. In determining effects of REG3β on insulin sensitivity, insulin tolerance test (ITT), glucose tolerance test (GTT), glucose stimulated insulin secretion (GSIS), plasma insulin assay (ELISA kit, Crystal Chem, Downers

Grove, IL) and plasma C-reactive protein (CRP) assay (ELISA kit, Cayman chemical, Ann Arbor, MI) were performed in fasting (overnight for 8 h) WT and KO mice (n = 6) as previously-described (Wang et al., 2008). In determining acute effects of recombinant REG3β protein administration on insulin sensitivity, a single ip injection of filter-sterilized REG3β protein at 2 μg/g body weight or equal volume [10 μl/g body weight (BW)] of PBS was given to the WT and KO male mice (n = 6) at 15 min prior to the administration of insulin (0.5 U of insulin/kg BW) and mice were euthanized within 5 min after the injection of insulin to collect tissues for immunoblotting analysis. In determining long-term effects of REG3β administration, daily ip injections of REG3β at 2 μg/g or PBS (control) were given to KO mice (n = 6) for 6 wk followed by ITT. In determining acute effects of inflammation on REG3β protein expression in the pancreas and intestine, and the subsequent impact on insulin sensitivity, WT and KO mice were given an ip injection of PBS or LPS (2 mg/kg BW) followed by ITT test at 12 hours after injections. Tissues and plasma were collected after ITT for qPCR, CRP, and immunoblotting analyses. In determining long-term effects of inflammation on REG3β protein expression in pancreas and intestine and the subsequent impact on insulin sensitivity, 2% DSS was added to drinking water for 2 wk followed by ITT at the end of the experiment. Tissues were collected after ITT for immunoblotting analysis. In determining effects of eliminating circulating REG3β protein in plasma on insulin sensitivity, 0.4 μg/g BW REG3β antibody or pre-immune rabbit serum (average BW, 25 g) were injected every other day for 2 wk followed by ITT at the end of the experiment. In determining acute effects of anti-inflammation drugs on REG3β protein expression in the pancreas and intestine, WT and KO mice were given an ip injection of PBS or

dexamethasone (DEX) (0.12 μg/g BW) followed by ITT at 6 h after injections. WT and KO mice were given an ip injection of aspirin (81 mg/kg BW, 10% DMSO) or statin (40 mg/kg BW, saline) 1 h prior to LPS injection (2 mg/kg BW) with drug solvent as control followed by ITT at 6 h after injections. Ibuprofen (25%) and sulindac (3%) were added to drinking water (to target a dose of 40 or 5 μg/g BW) for 4 wk to mice that were injected 2 mg/kg BW LPS every other day. Tissues from these studies were collected for immunoblotting analysis. In determining acute effects of recombinant REG3α protein administration on insulin sensitivity, a single ip injection of filter-sterilized REG3α protein at 4 μg/g BW or equal volume (10 μl/g BW) of PBS was given to the WT and KO male mice (n = 6) at 15 min prior to ITT.

2.2.2 Q-PCR, Immunobloting, immunohistochemistry and H&E staining, hepatocyte isolation, culture, and treatments.

Q-PCR and immunoblotting were performed as previously described with primers and antibodies listed in *Appendix* Table 1 and 2 (Yan et al., 2012). The whole pancreas and intestine tissue were removed from mice, fixed in 4% paraformaldehyde at 4°C overnight, washed 3 times in PBS, transferred into 70% ethanol, and placed into a cassette for embedding (Sund et al., 2001). The embedded samples were sectioned at 5 μm, and the slides were rehydrated through xylenes and decreasing grades of alcohol and stained (H & E) at the Cornell histology facility (Ithaca, NY). Staining was viewed using an Axiovert 200 M fluorescent microscope (Zeiss, Carl-Zeiss-Strasse, Oberkochen, Germany). The primary hepatocytes were isolated from the WT and KO mice by type IV

collagenase (2 mg/mL, Sigma-Aldrich) digestion of liver following a standard protocol with minor modifications (Severgnini et al., 2012). The isolated hepatocytes were then cultured in William Medium E (WME) media for 12 h at 37°C (5% CO_2) before being treated with 20 ng/mL REG3β protein for 6 h. Hepatocytes untreated with REG3β served as a negative control. Relative mRNA level of *Insr*, *Akt1-3, C/ebpb, Nr3c1, Hnf3b, Wt1* and *C/ebpd* was measured by Q-PCR.

2.2.3 Heterologous expression and purification of native Reg3β and Reg3α proteins

The NOD mouse *Reg3β* cDNA (NM_011036), human *Reg3α* cDNA (NM_002580) human *Cxcl12* (NM_000609) or human *Il6* (NM_000600) were inserted into the *Pichia pastoris* expression vector pPICZ (Invitrogen, Grand Island, NY) in a way that allowed expression of REG3β, REG3α, CXCL12 and IL6 with His-tag at the C-terminus and without the respective intrinsic signal peptide sequences at the N-terminus using XhoI and EcoRI or SacI and XhoI restriction sites. This construct and vector pPICZαC were digested with BstBI and EcoRI, and then the *Reg3β*, *Reg3α, Cxcl12 or Il6* - fragment was ligated into the pPICZ vector backbone to create *Reg3β* or *Reg3α* - pPICZαA.

Chemically competent *E. coli* was transformed via heat shock (Froger and Hall, 2007). Sequence verification was performed by Big Dye Terminator chemistry at the Cornell Biotechnology Resource Center (Ithaca, NY). Isolated *Reg3β* or *Reg3α* plasmids were transformed into the yeast *P. pastoris* GS200 using electroporation (BTX, Holliston, MA) (Lin-Cereghino et al., 2005), and cultured in BMMY media for 3 d. Thereafter, the

culture supernatant was collected and brought to 40% saturation with ammonium sulfate to precipitate the REG3β or REG3α proteins (Yamada et al., 2012). The precipitates were dissolved in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole at pH 8.0, and then dialyzed against the same buffer. Next, the dialyzed solutions were subjected to Ni-NTA column (Qiagen, Hilden, Germany) following manufacture instructions and the purity of the target proteins was verified by SDS-PAGE and immunoblotting. The REG3β or REG3α containing fractions were pooled, concentrated by 40% ammonium sulfate precipitation, and dissolved in and dialyzed against PBS at pH 7.4.

2.2.4 Putative transcription factor binding sites and Chromatin immunoprecipitation (ChIP) assay

The proximal promoter regions of *Insr*, *Akt1,* and *Akt2* defined as -1,000/1,100 bp upstream of the transcription start site, were retrieved from the NIH/NCBI Entrez Gene database (http://www.ncbi.nlm.nih.gov/gene). The transcription factor binding sites were predicted using the Transcription Element Search System (TESS, http://www.cbil.upenn. edu/cgi-bin/tess/tess).

ChIP assay was performed using Imprint Chromatin Immunoprecipitation Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. Chromatin isolated from the WT and KO hepatocytes was immune-precipitated with antibodies against NR3C1 (Novus Biologicals, Littleton, CO) (*Appendix* Table 2) or mouse IgG (Sigma-

Aldrich, St. Louis, MO). Three or four sets of primers covering the proximal regions of the *Insr, Akt1* and *Akt2* promoters were used for PCR analysis (*Appendix* Table 1).

2.2.5 Luciferase reporter assays of the *Insr* and *Akt1-2* gene promoter

Various promoter regions of *Insr* and *Akt1-2* (-1100/-1000 to 0, -500 to 0, -250 to 0) were amplified using forward and reverse primers adding restriction sites (underlined) - Kpn I and Nhe I (New England Biolabs, Ipswich, MA) for cloning into corresponding sites of pGL3-basic luciferase reporter vector (Promega, Madison, WI) (*Appendix* Table 1). Constructed *Insr* and *Akt1-2* promoter-luciferase reporter vectors were characterized by restriction analysis and DNA sequencing. The PCR protocol was 95ºC for 7 min followed by 35 cycles of 95ºC for 30 sec, 64ºC for 30 sec, and 72ºC for 1.5 min. The PCR products were purified using Wizard SV gel and PCR cleanup columns (Promega, Madison, WI), digested by aforementioned restriction enzymes, and ligated into the pGL3-basic luciferase reporter vector by T_4 DNA ligase (Invitrogen, Grand Island, NY). The ligated vector was transformed into *E. coli* DH5α competent cells using heat shock (Froger and Hall, 2007).

HepG2 cells were grown in WME media supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1% PenStrep, 0.4% fungizone, 5 μg/ml insulin, 1 μg/ml glucagon (Gibco, Grand Island, NY) and 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO) at 37ºC in 5% CO_2 atmosphere. HepG2 cells were seeded in 6-well plates, grown to 80–90% confluence, and transfected by Lipofectamine^{TM} 2000 (Invitrogen, Grand Island,

NY) according to the manufacturer's instructions. Cells were transfected with the pGL3-basic luciferase reporter vectors with the proximal promoters of *Insr* and *Akt1-2* and Renilla luciferase control reporter vector (Promega, Madison, WI) for transfection efficiency normalization. After 24 h of transfection, cells were treated with 20 ng/ml REG3β for 12 h, and then were harvested in passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) on a Berthold Lumat LB 9501 (Berthold, Wildbad, Germany).

2.2.6 Constructs of REG3β protein conjugated with enhanced green fluorescent protein (EGFP) (*Reg3β-EGFP*), CXCR4 protein conjugated with mCherry (*Cxcr4-mCherry*), and EXTL3 protein conjugated with mCherry (*Extl3-mCherry*)

For testing the binding between REG3β and CXCR4 or EXTL3, the NOD mouse *Reg3β* cDNA (NM_011036), mouse *Cxcr4* (NM 001356509) and mouse *Extl3* (NM 001360385) were inserted into the PcDNA 3.0-EGFP vector or pmCherry-C3 vector (*Appendix* Tables 1 and 3) in a way that allowed expression of REG3β, CXCR4, and EXTL3 with EGFP or mCherry tag at the N or C-terminus using XhoI and HindIII, XhoI and SalI or HindIII and KpnI restriction sites. These constructs were used for co-immunoprecipitation (Co-IP) and fluorescent co-localization assay.

2.2.7 Co-immunoprecipitation

After REG3β-EGFP was overexpressed extracellularly in the HepG2 cells for 48 h and bound with intrinsic CXCR4 in the presence of CXCL12-His (produced in yeast) or intrinsic GP130 in the presence of IL6-His (produced in yeast) added into the medium, the binding complex was cross-linked by 1 mM disuccinimidyl suberate (DSS) (Thermo Scientific, Waltham, MA) for 30 min according to the manufacturer's instructions. Thereafter, the binding complex was pulled-down by the GFP-trap beads (chromotek, Planegg, Germany) or mouse IgG and protein G beads (Santa Cruz, Dallas, TX) (Zhong et al., 2007) in dilution buffer containing 150 mM NaCl, 0.5 mM EDTA and 10 mM Tris-HCl (pH=7.5), at 4°C overnight. Samples were washed 3 times with ice-cold dilution buffer, and proteins were eluted using an SDS-PAGE loading buffer at 100°C for 5 min (Cheng et al., 2003). After centrifugation, supernatant was loaded into SDS-PAGE gels for immunoblotting detection with CXCR4 or GP130/His-tag/REG3β antibodies (*Appendix* Table 2).

After EXTL3-mCherry was overexpressed in the HepG2 cells for 48 h and bound with REG3β protein (produced in yeast) added into the medium, the binding complex was pulled down by an EXTL3 antibody or mouse IgG with protein G beads (Santa Cruz, Dallas, TX) in buffer containing 20 mM Tris HCl (pH = 8), 137 mM NaCl, 1% nonidet P-40 (NP-40), 2 mM EDTA (pH=7.5) at 4°C overnight. Samples were washed 5 times with ice-cold buffer, and proteins were eluted using an SDS-PAGE loading buffer at 100°C for 5 min (Germino et al., 1993). After centrifugation, supernatant was loaded into SDS-PAGE gels for immunoblotting detection with REG3β antibody (*Appendix* Table 2).

2.2.8 Co-localization of REG3β-EGFP with CXCR4-mCherry or EXTL3-mCherry

HEK293T cells (1×10^5 cells/mL) were seeded the day before transfection. The *Reg3β*-EGFP or GFP vectors were then transfected into the HEK293T cells by using PEI (Boussif et al., 1995). The cells were incubated in DMEM media with 10% FBS, 1% PenStrep at 37°C with 5% CO_2. After 3 to 4 d, conditioned media (CM) was collected for the co-localization assay. REG3β-EGFP protein expression was measured by using an Axiovert 200 M fluorescent microscope (Zeiss, Carl-Zeiss-Strasse, Oberkochen, Germany). Human HepG2 cells were transfected with plasmid DNA of *Extl3*-mCherry or *Cxcr4*-mCherry by using lipofectamine^{TM}2000 (Invitrogen) according to the manufacturer's instructions. After 48 h of incubation, the cells were washed with HBH containing 137 mM NaCl, 5.4 mM KCl, 0.4 mM $MgSO_4*7H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2*2H_2O$, 0.3 mM $Na_2HPO_4*7H_2O$, 20 mM HEPES (pH 7.4), and 0.1% BSA twice and then incubated with CM for 2 h at room temperature (Truong and Ikura, 2001). Cells were further washed with ice cold HBH for 3 times and then images of fluorescences were captured by using a Zeiss 880 Confocal-MP Inverted microscope (Zeiss, Carl-Zeiss-Strasse, Oberkochen, Germany).

2.2.9 siRNA knockout and 1,9-Dimethylmethylene Blue (DMB) assay

After 12 h seeding of HepG2 cells in 6-well plates at density (7.5×10^5 cells/mL), siRNA (Santa Cruz, Dallas, TX) against *Extl3* or *Nr3c1* and control siRNA were transfected to cells with lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Transfection reagent was removed after 12 h incubation with

complete WME medium. After 36 h of incubation, cells were either collected for qPCR analysis or treated with 20 ng/mL REG3β for 6 h before being processed for qPCR analysis or dimethylmethylene blue (DMB) binding assay.

DMB binding assays were performed to quantify the levels of total glycosaminoglycans (GAG) chains on the HepG2 cell surface. Approximately 1×10^5 cells from each treatment were collected in 200ul PBS. Then, DMB (250 µL/well, Sigma-Aldrich, St. Louis, MO) was added to 25 µL of sample and quantified at 520 nm.

2.2.10 Statistical analysis

Quantitative data of genotypic differences and various treatment effects were analyzed using one-way ANOVA with or without time-repeated measurements (SAS 6.11 software, SAS Institute, Cary, NC). Values were expressed as mean ± SEM. Significance was defined as $P < 0.05$ unless indicated otherwise.

CHAPTER THREE

RESULTS

3.1 Knockout of REG3β enhanced insulin sensitivity and inhibited the inflammation induced insulin resistance

The exceptional baseline insulin sensitivity in the REG3β$^{-/-}$ mice was initially shown by their "glucose shock" after an injection (ip) of a regular dose of insulin [0.5 units (U)/kg]. A half dose (0.25 U/kg) led to 2.3-7 fold greater decreases ($P < 0.05$) of blood glucose at 30 and 60 min in the REG3β$^{-/-}$ than wild-type mice (WT) at both young (2-month) and old (10-month) ages (Fig. 3.1 and 3.2). Consistently, the REG3β$^{-/-}$ mice had 17.5-30.8% lower ($P < 0.05$) plasma glucose concentrations (Fig. 3.3) and 59% lower ($P < 0.05$) glucose-stimulated insulin secretion (Fig. 3.4) than the WT, despite similar body weights (Fig. 3.5). More convincingly, to determine the physiological relevance between improved insulin sensitivity and REG3β deletion, we administered KO mice multiple bi-daily injections of the purified REG3β protein overexpressed and purified from the yeast and the knockout resultant enhancement of insulin sensitivity was reversed by exogenous REG3β administrations (2 μg/g of body weight, ip injection) for 6wk (Fig. 3.6 and 3.7). Therefore, REG3β acted as a specific, potent, and negative determinant of the baseline insulin sensitivity. Most strikingly, treating both genotypes with 2mg/kg BW LPS by i.p. injection 12 h prior to performing an insulin tolerance test (ITT, 0.25U/kg BW) or 2% DSS in the drinking water for 2 wk induced IR (29%) only in the WT (Fig. 3.8 and 3.9).

The LPS or DSS induced IR concurred with a substantial elevation of REG3β ($P< 0.05$) in the pancreas and intestine (Fig. 3.10 and 3.11). Although inflammation was produced by LPS and DSS in *both* genotypes, as confirmed by tissue pathology shown as more gaps in the pancreas and looser connections between cells and watery fecal in the intestines (H&E staining and dissection observation) and plasma C-reactive protein (CRP) elevation (Fig. 3.12-3.14), knockout of REG3β precluded the resultant inflammation from inducing IR in the mice.

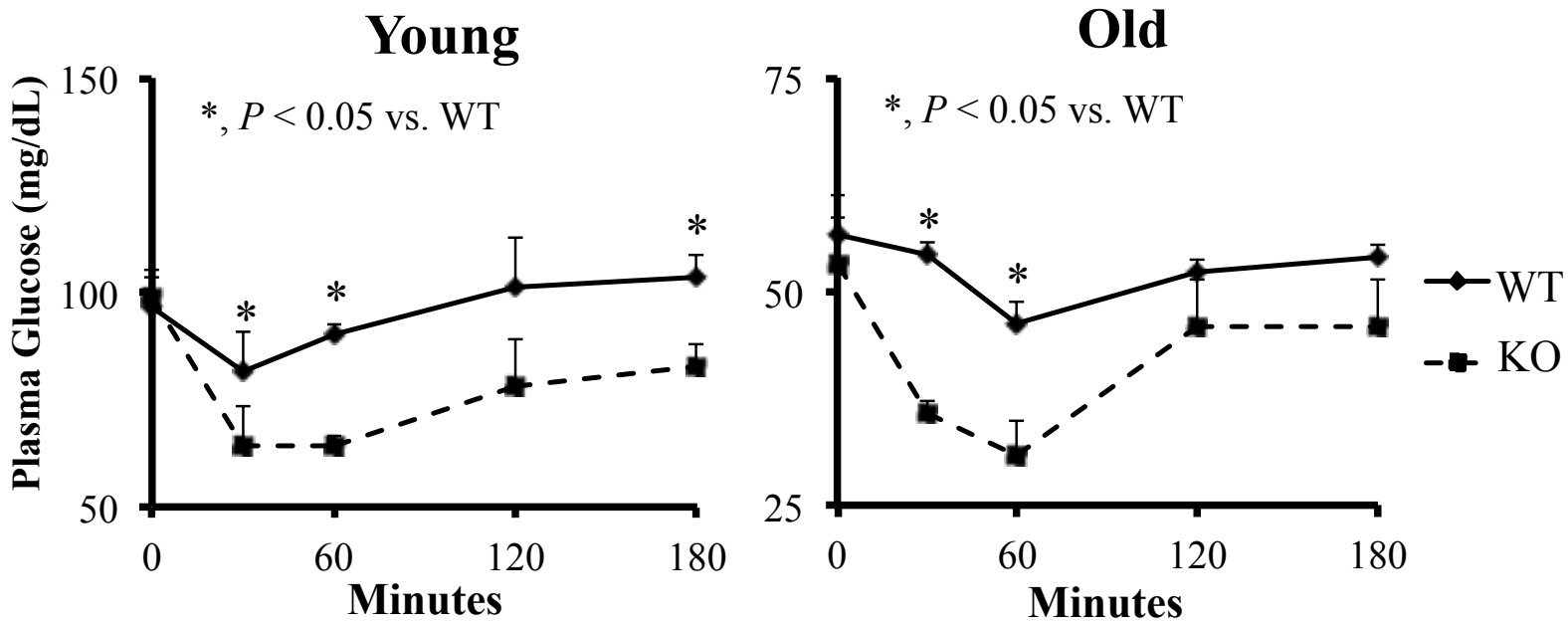

Figure 3.1 Enhanced insulin sensitivity, as shown by the insulin tolerance test (ITT, 0.5 U/kg) in the KO compared with the WT mice after 8 h overnight fasting at 2- and 10-month ages. Data are mean ± SE (n = 6-10) *, $P < 0.05$ vs. WT. Also refer to Figure 3.2 to 3.5.

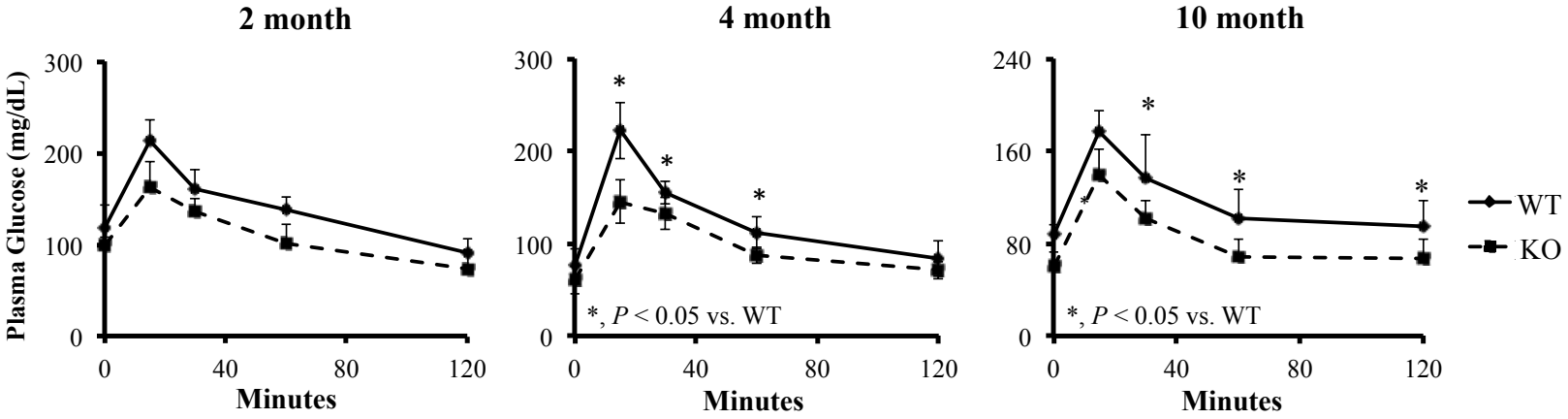

Figure 3.2 Improved glucose tolerance, as shown by the glucose tolerance test (GTT, 1 g/kg) in the KO compared with the WT mice after 8 h overnight fasting at 4- and 10-month ages. Data are mean ± SE (n = 6-10), and *, $P < 0.05$ vs. WT. Also refer to Figure 3.1.

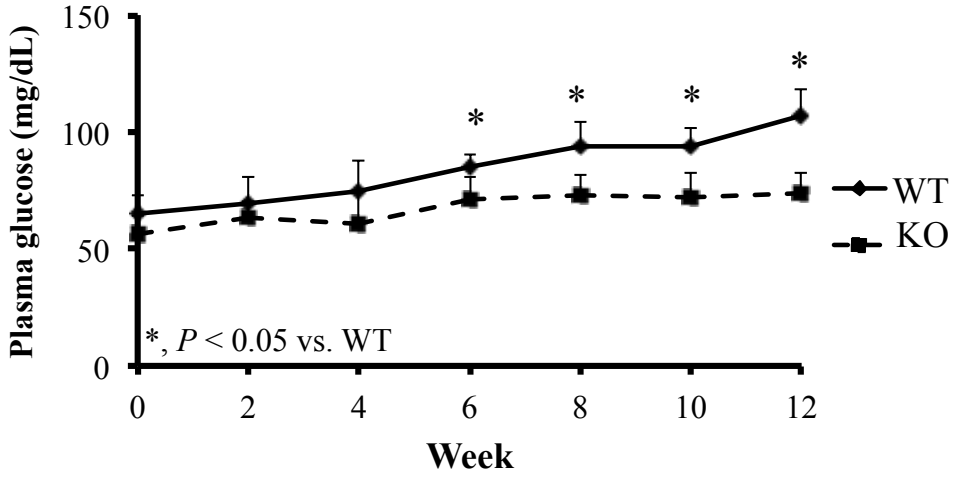

Figure 3.3 Decreased plasma glucose as shown by biweekly measurements for 12 wk in the KO compared with the WT mice (2 month old) after 8 h overnight fasting. Data are mean ± SE (n = 6), and *, $P < 0.05$ vs. WT. Also refer to Figure 3.1.

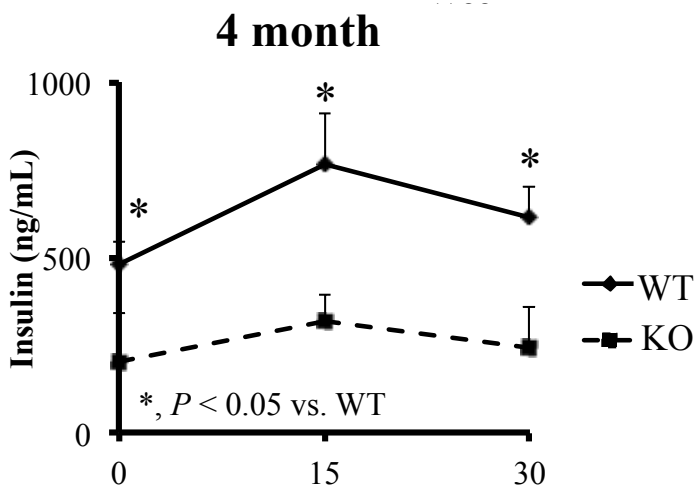

Figure 3.4 Improved glucose stimulated insulin secretion, as shown by plasma insulin concentrations after the stimulation by 1g/kg glucose injection in the KO compared with the WT mice after 8 h overnight fasting at 5-month ages. Data are mean ± SE (n = 6-10), and *, $P < 0.05$ vs. WT. Also refer to Figure 3.1.

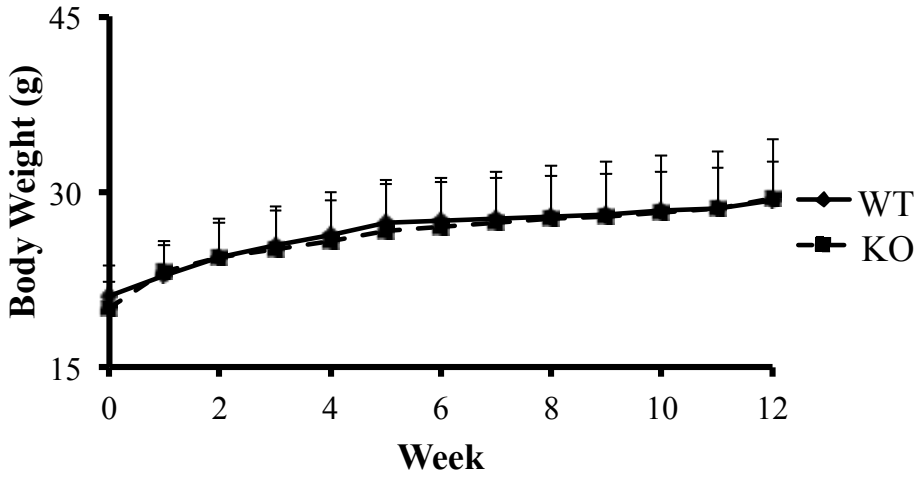

Figure 3.5 No difference in body weight, as shown by biweekly measurements for 12 wk in the KO compared with the WT mice (2 month old) after 8 h overnight fasting. Data are mean ± SE (n = 6-10), and *, $P < 0.05$ vs. WT. Also refer to Figure 3.1.

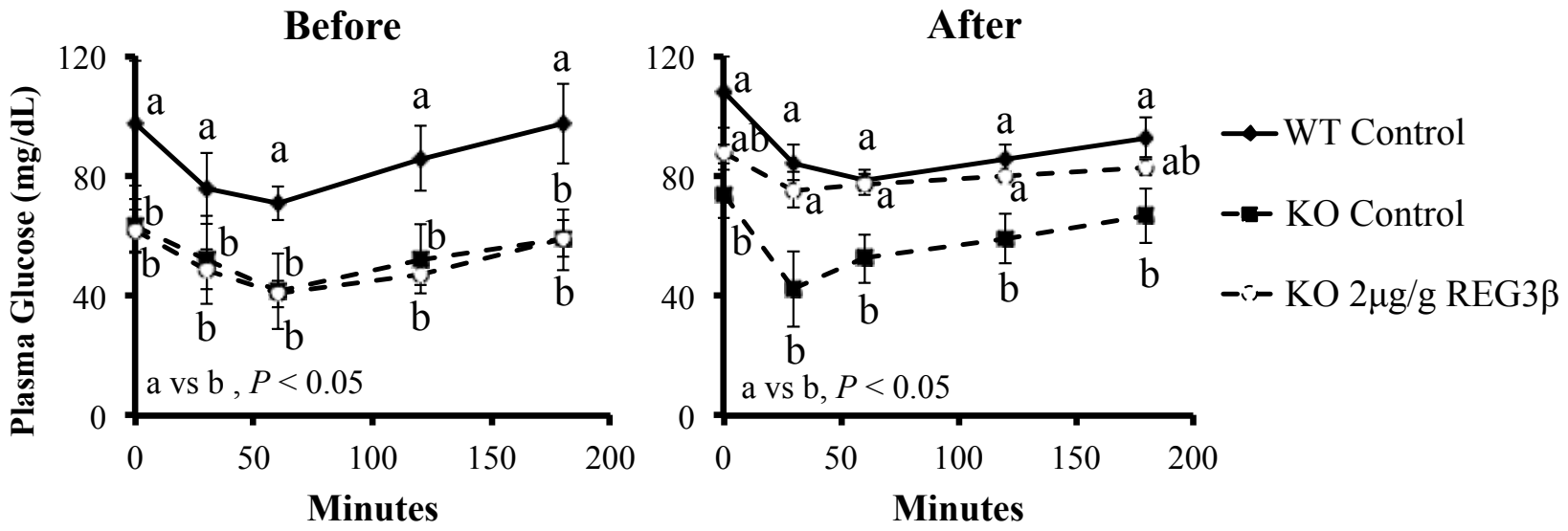

Figure 3.6 Decreased insulin sensitivity (0.5 U/kg) in the KO mice by daily i.p. injections of the native REG3β protein (2 μg/g body weight) for 6 wk compared with the PBS-injected WT and KO mice. Data are mean ± SE (n = 10), and *, $P < 0.05$ vs PBS. Also refer to Figure 3.7.

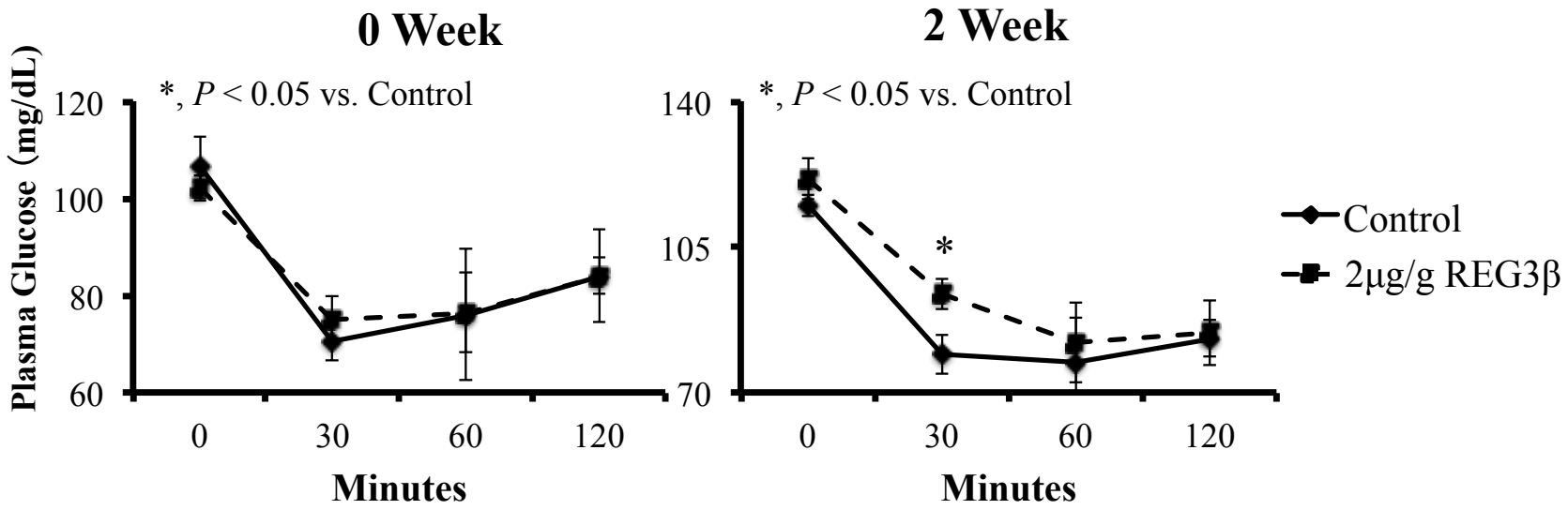

Figure 3.7 Decreased insulin sensitivity, as shown by ITT (0.5 U/kg), in the KO mice by daily i.p. injections of the native REG3β protein (2 μg/g body weight) for 2 wk compared with the PBS-injected WT and KO mice. Data are mean ± SE (n = 5), and *, $P < 0.05$ vs PBS. Also refer to Figure 3.6.

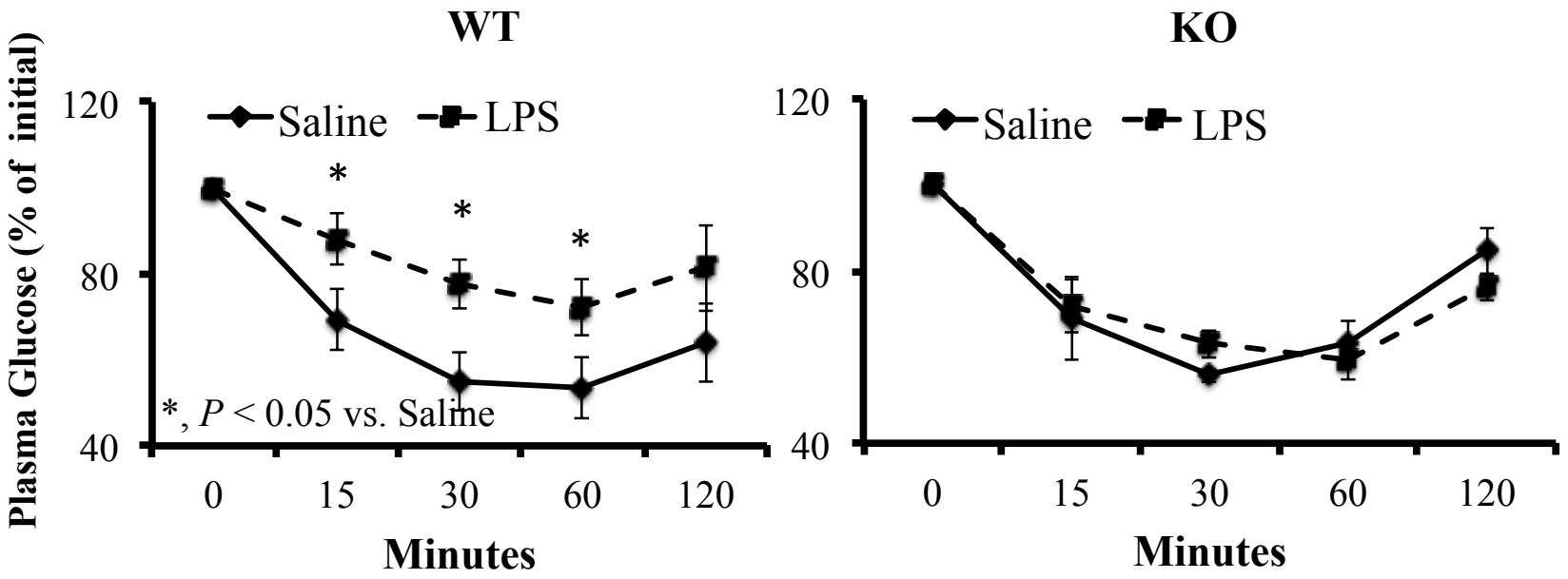

Figure 3.8 Induced insulin resistance at 12 h after the injection of LPS (2 mg/kg), as shown by ITT, in the WT mice (1st panels) but not in the KO mice (2nd panels). Data are mean ± SE (n = 6), *, $P < 0.05$ vs. saline. Also refer to Figure 3.9.

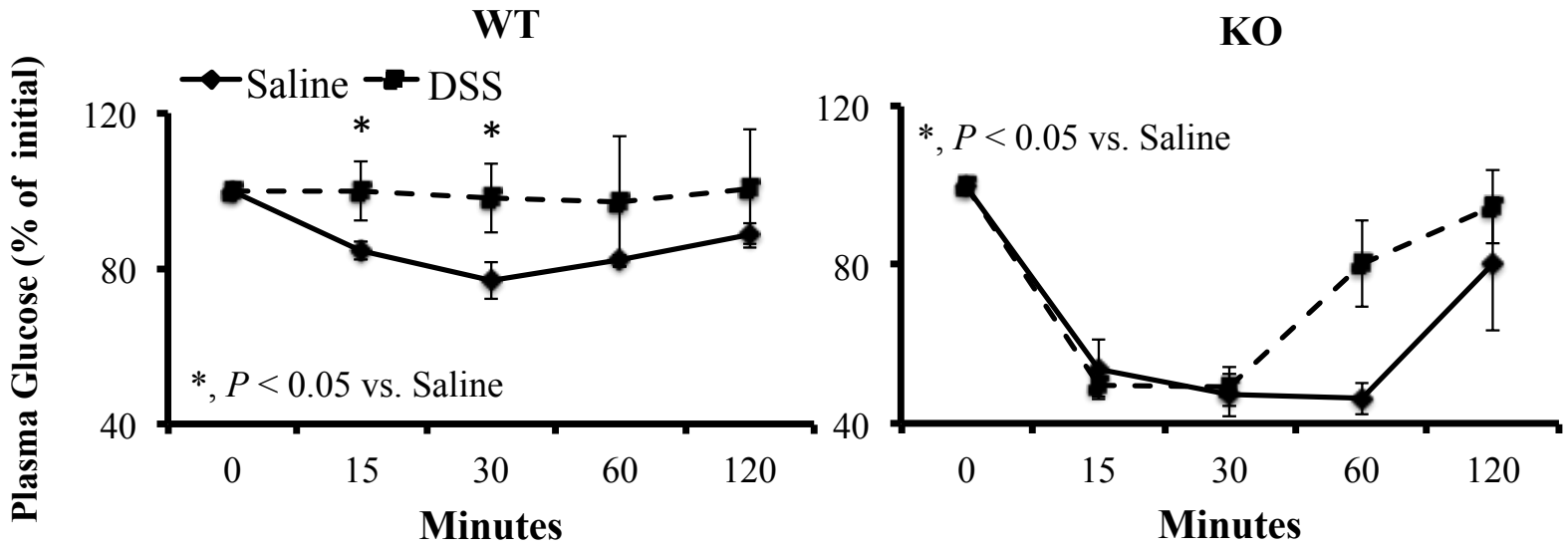

Figure 3.9 Induced insulin resistance at 2 wk after the administration of 2% DSS in the drinking water, as shown by ITT, in the WT mice (left panel) but not in the KO mice (right panel). Data are mean ± SE (n = 6), and *, $P < 0.05$ vs. saline data are mean ± SE (n = 6), *, $P < 0.05$ vs. WT. Also refer to Figure 3.8.

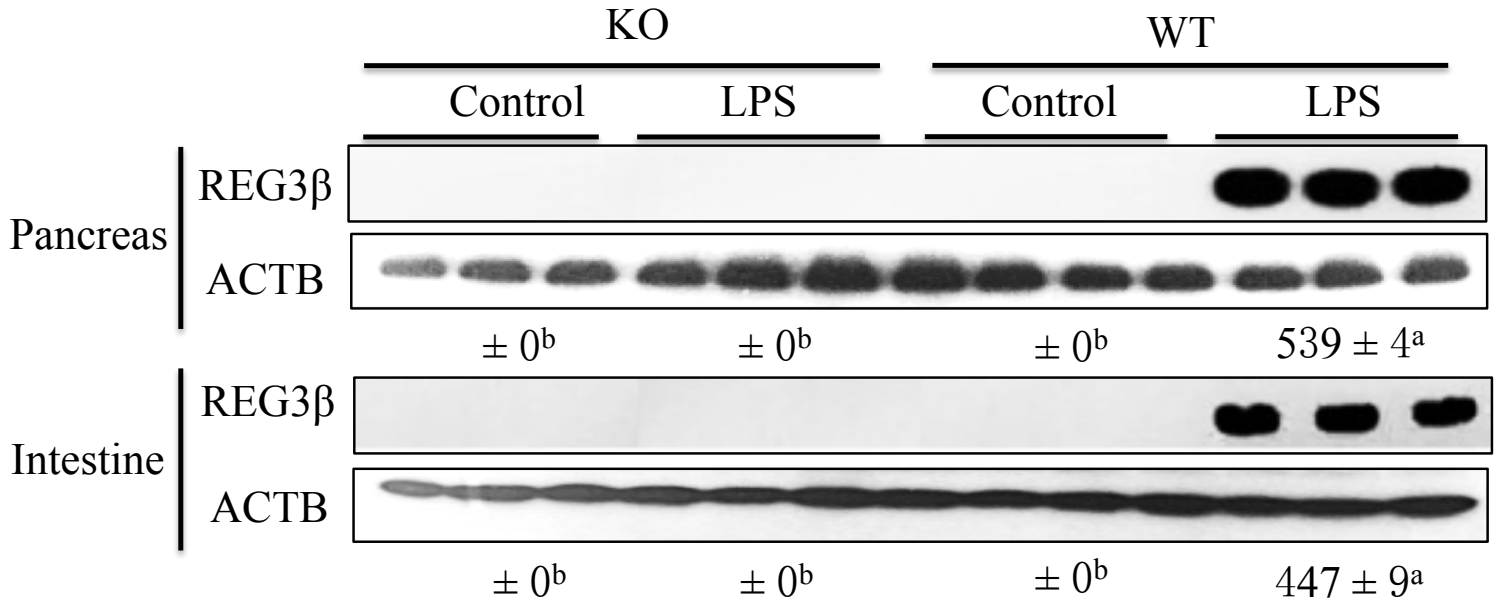

Figure 3.10 Induced REG3β protein production, shown by immunoblotting, in the pancreas and intestine of WT, but not in the KO mice at 12 h after the injection of LPS. Data are mean ± SE (n = 6) and normalized to β-ACTIN as the loading control, *, $P < 0.05$ vs. control. Also refer to Figure 3.11

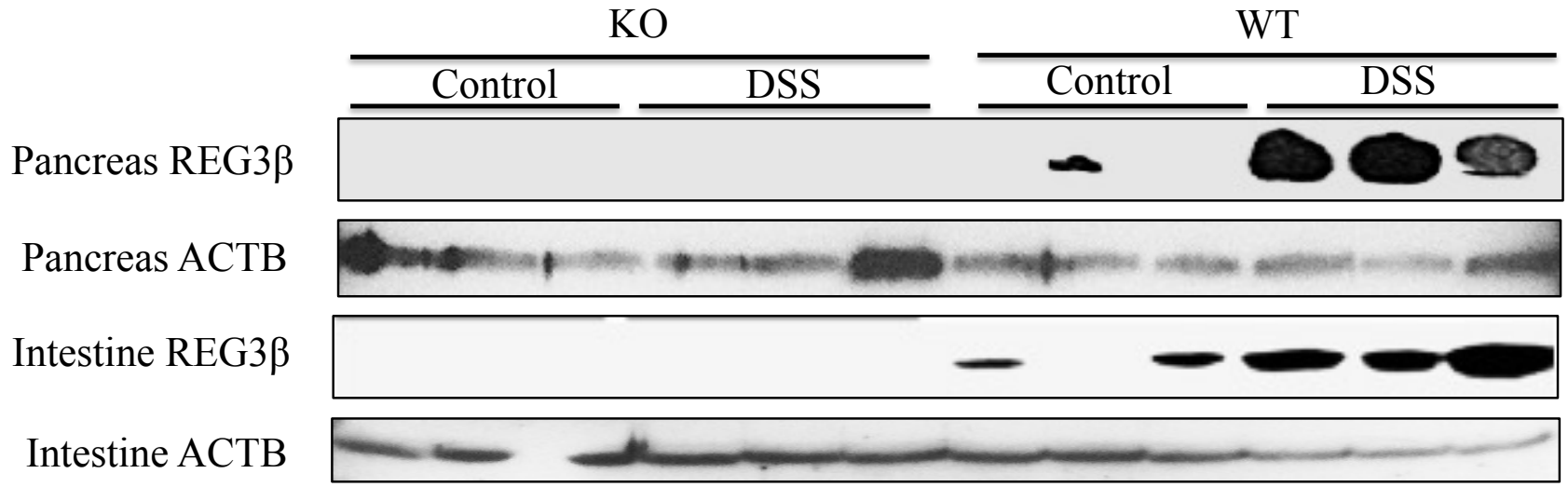

Figure 3.11 Elevated REG3β protein production, shown by immunoblotting, in the pancreas and intestine of WT, but not in the KO mice at 2 wk after the administration of 2% DSS in drinking water. Data are mean ± SE (n = 6) and normalized to β-ACTIN as the loading control, and *, $P < 0.05$ vs. control. Also refer to Figure 3.10.

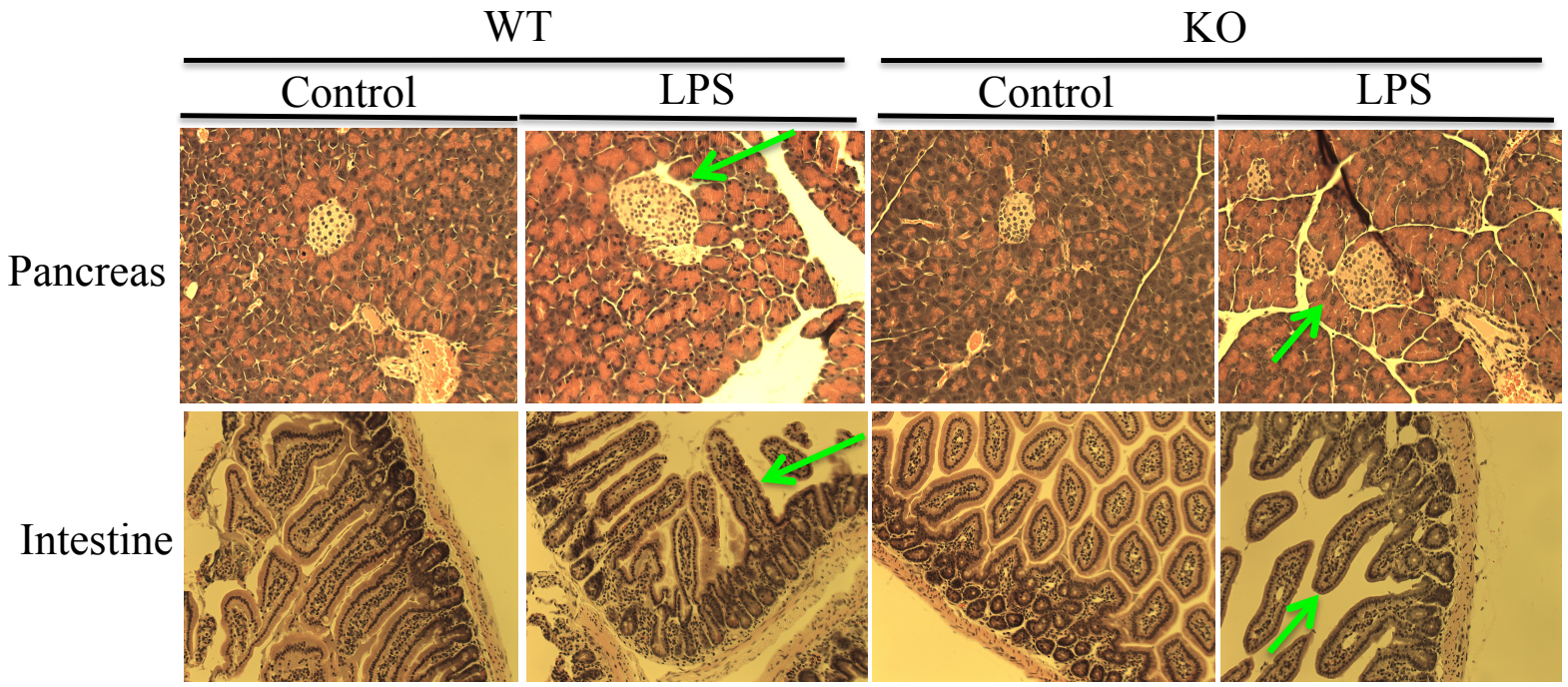

Figure 3.12 H&E staining of the pancreas (upper panel) and intestine (lower panel) of both genotypes to show the LPS-injection (after 12 h) induced inflammation. The image was a representation of 4 independent assays. Also refer to Figure 3.14.

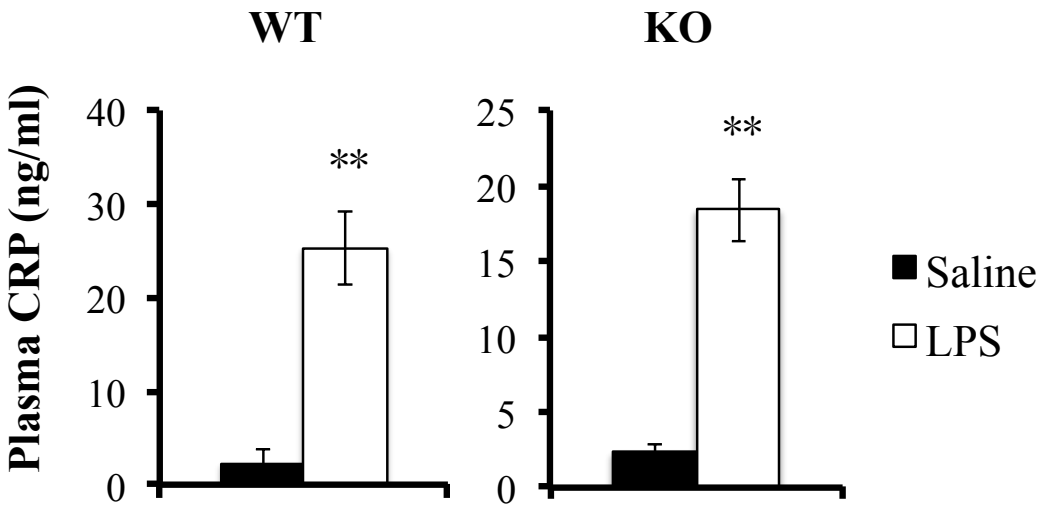

Figure 3.13 Elevated plasma C-reactive peptide by the LPS injection (after 12 h) in both WT and KO mice. Data are mean ± SE (n = 6) **, $P < 0.01$ vs. respective saline controls. Also refer to Figure 3.14;

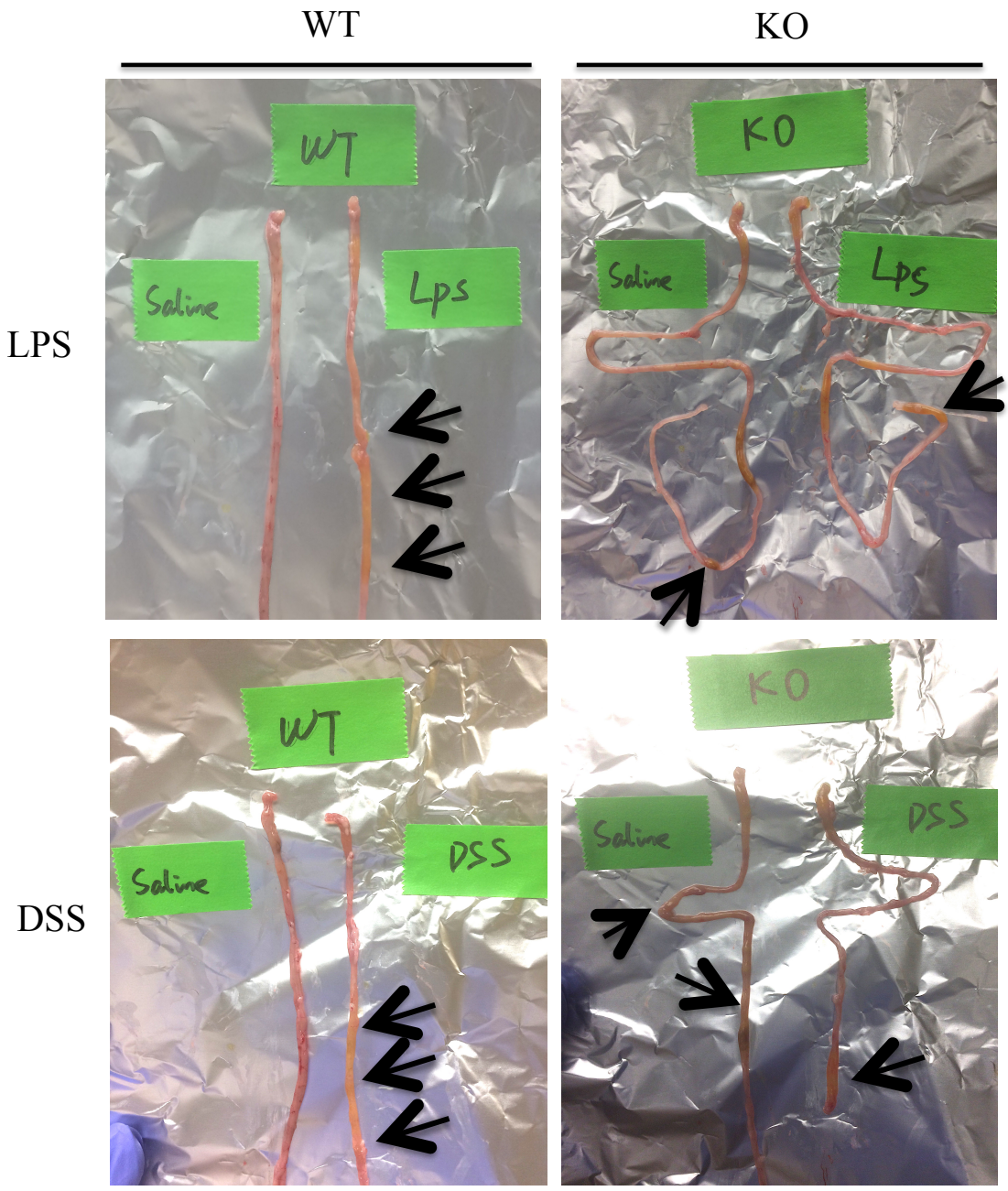

Figure 3.14 Dissecting the intestine of both genotypes to show the LPS-injection (after 12 h) induced inflammation. Arrows define the spot that demonstrates inflammation. The image was a representation of 4 independent assays. Also refer to Figure 3.12 and 3.13.

3.2 REG3β regulated INSR and AKT expression and activation

To understand the enhanced baseline insulin sensitivity by knockout of REG3β, we determined the status of key insulin signal proteins in the two genotypes. qPCR and immunoblotting analysis illustrated that the REG3β$^{-/-}$ mice had 70% to 170% elevated gene transcripts ($P < 0.05$) (Fig. 3.15), 31% to 139% enhanced total protein, and 39% to 92% increased phosphorylation of INSR and AKT (Fig. 3.16) in the liver and muscle compared with WT mice whereas phosphatase and tensin homolog (*Pten*), phosphoinositide 3-kinase (*Pi3k*), *Irs1,* and *Irs2* were excluded for remaining little or no change in KO mice (Fig. S3.17). The 15 mins prior administration (i.p. injection) of recombinant REG3β protein from yeast to the administration of 0.25U/kg BW insulin to WT and KO mice suppressed (56% to 99%, $P < 0.05$) insulin-stimulated phosphorylation of INSR and AKT in the two tissues of both genotypes (Fig. 3.18). The specific inhibition ($P< 0.05$) of *Insr* and *Akt1-3* gene expression by the exogenous 20ng/ml REG3β treatment for 12 hr was also shown in primary hepatocytes (54 to 83%) (Fig. 3.19) and HepG2 cells (Fig. 3.20). These data indicated that the knockout of REG3β enhanced baseline insulin sensitivity via up-regulating the tissue expression of INSR and AKT and their phosphorylation.

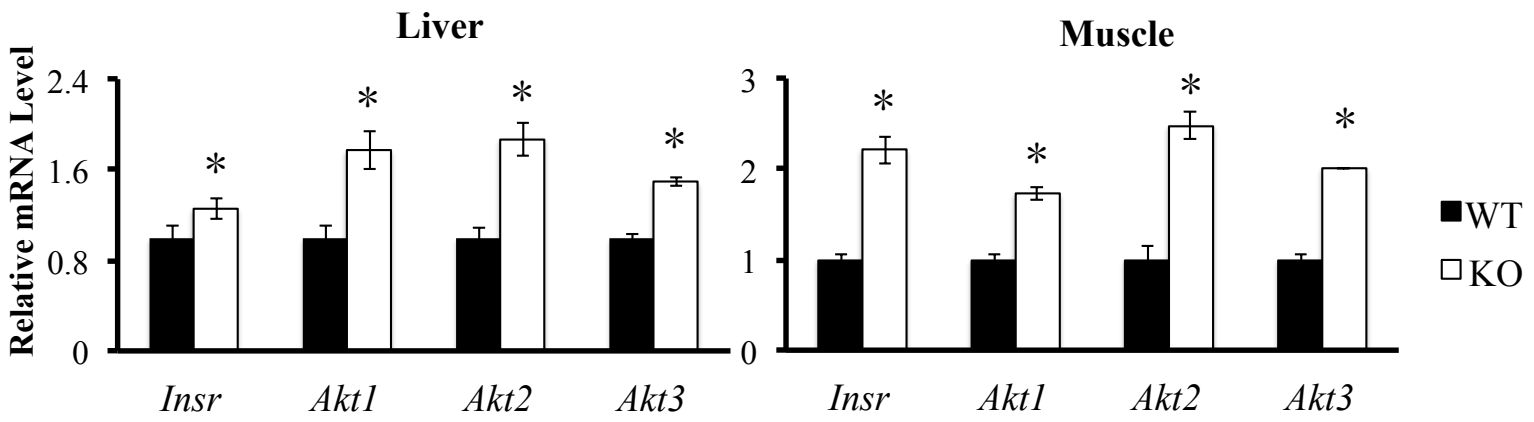

Figure 3.15 Up-regulated mRNA levels of *Insr* and *Akt1-3* in the liver (left panel) and muscle (right panel) of the KO compared with the WT mice. Data are mean ± SE (n = 6-10) and normalized to the *β-actin* mRNA levels, *, $P < 0.05$ vs. WT. Also refer to Figure 3.17.

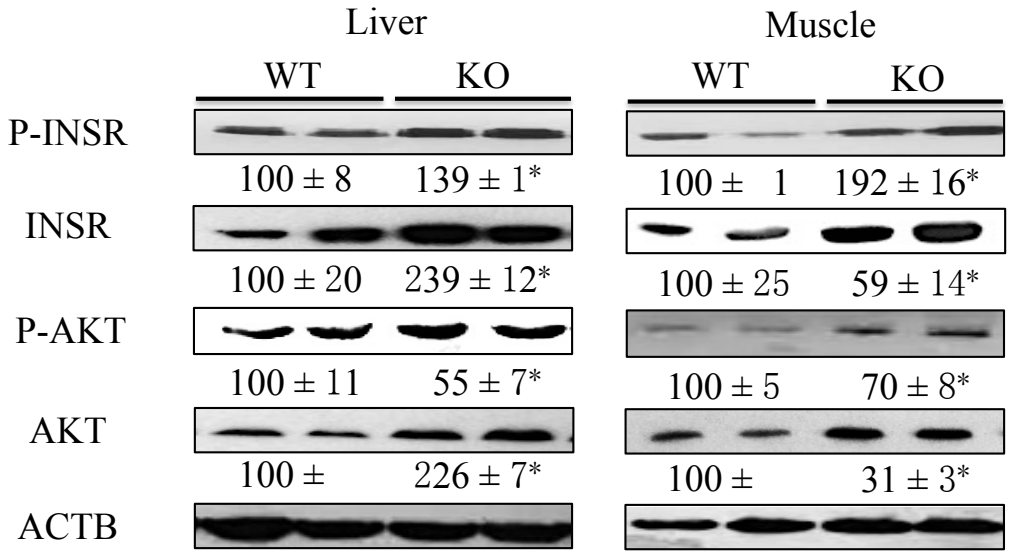

Figure 3.16 Increased phosphorylated and total INSR and AKT proteins, as shown by immunoblotting, in the liver (1st panel) and muscle (2nd panel) of the KO compared with the WT mice. Data are mean ± SE (n = 5) and normalized to β -Actin as the loading controls, *, $P < 0.05$ vs. WT.

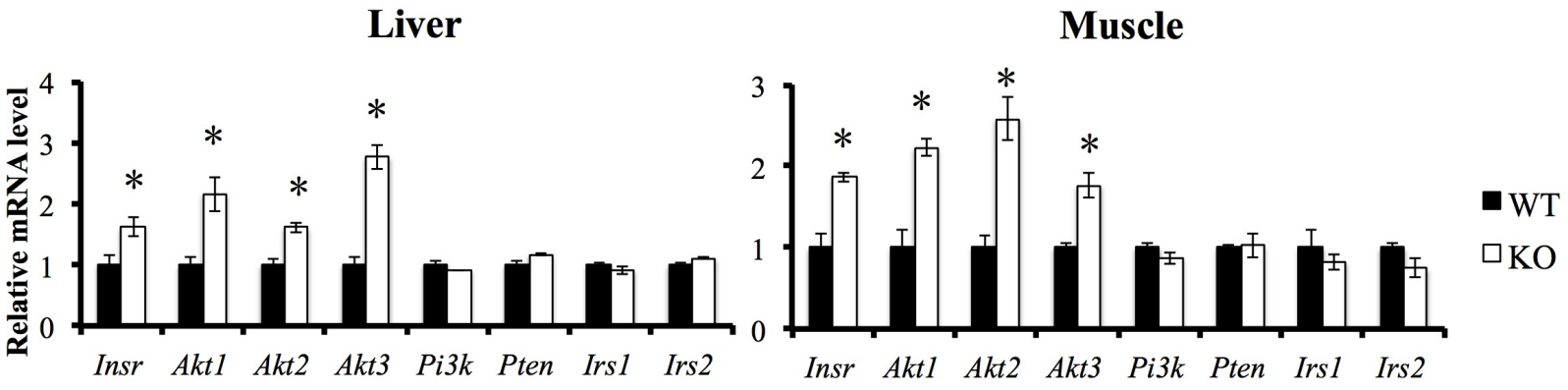

Figure 3.17 Up-regulated mRNA levels of *Insr* and *Akt1-3* but not *Pten, Pi3k,* or *Irs1* and *2* in the liver (left panel) and muscle (right panel) of the KO compared with the WT mice. Data are mean ± SE (n = 6-10) and normalized to the *β-actin* mRNA levels. *, $P < 0.05$ vs. WT. Also refer to Figure 3.15.

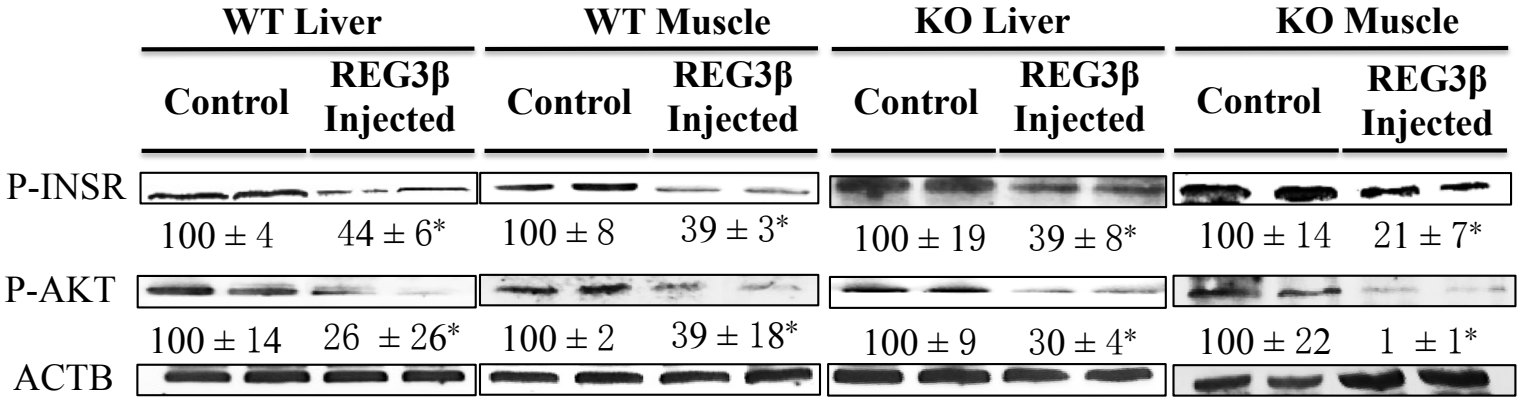

Figure 3.18 Inhibited phosphorylation of INSR and AKT proteins, as shown by immunoblotting, in the liver (1st panel) and muscle (2nd panel) of the WT mice and the liver (3rd panel) and muscle (4th panel) of the KO mice by an i.p. injection of the native REG3β protein (2 μg/g body weight) compared with the PBS-injected WT and KO mice at 15 min prior to the ITT (0.5 U/kg). Mice were killed to 3 and 7 min after the injection of insulin to collect liver and muscle samples for analysis, respectively. Data are mean ± SE (n = 5) and normalized to ß-actin as the loading controls, *, $P < 0.05$ vs. WT.

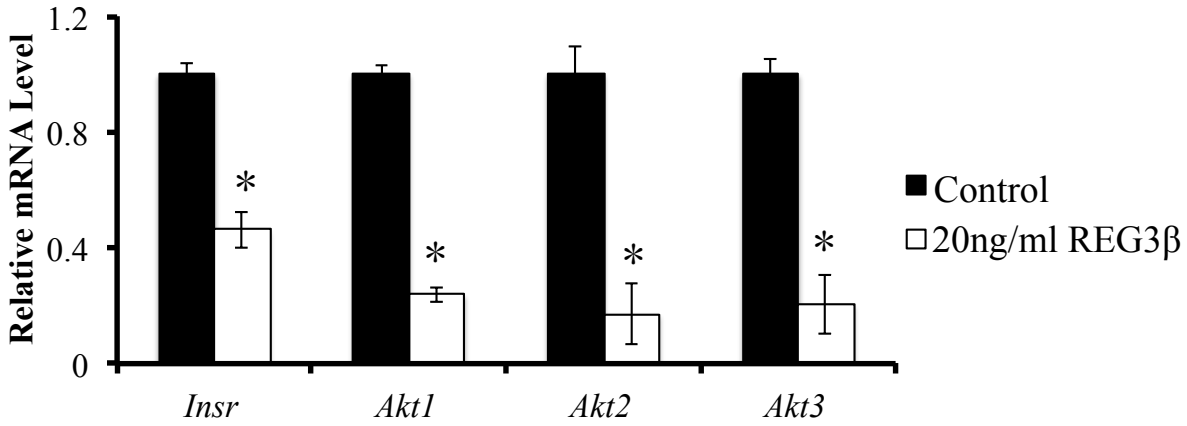

Figure 3.19 Suppressed mRNA levels of *Insr* and *Akt1-3* in the primary hepatocytes of KO mice by treating with REG3β (20 ng/mL) for 6 h compared with the PBS treatment. Data are mean ± SE (n = 5) and normalized to the *β-actin* mRNA levels, *, $P < 0.05$ vs. control. Also refer to Figure 3.20.

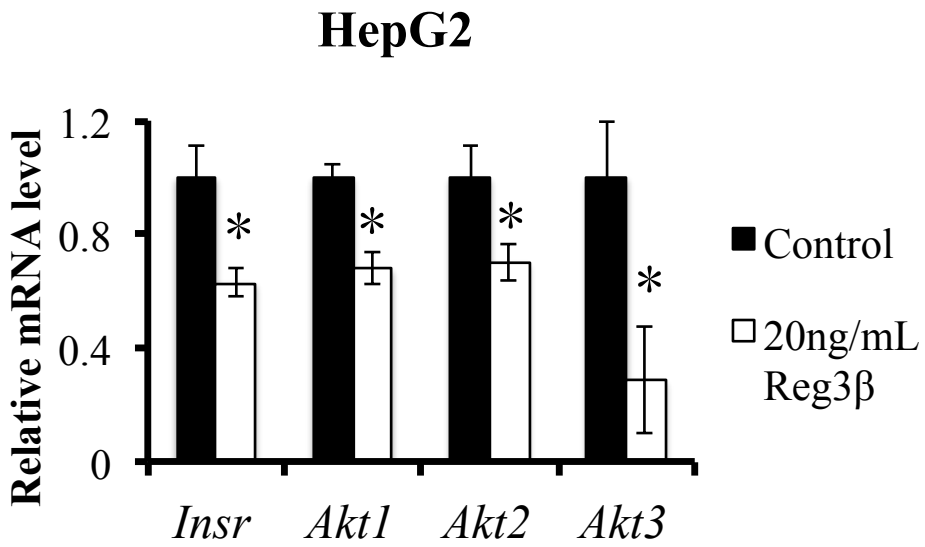

Figure 3.20 Suppressed mRNA levels of *Insr* and *Akt1-3* in the HepG2 cells by treating with REG3β (20 ng/mL) for 6 h compared with the PBS treatment. Data are mean ± SE (n = 5) and normalized to the *β-actin* mRNA levels. *, $P < 0.05$ vs. control. Also refer to Figure 3.19.

3.3 The binding of REG3β to transmembrane EXTL3 stimulated the binding of NR3C1 to the proximate promoters of *Insr* and *Akt1* and *2*, resulting in transcriptional inhibition of these genes

To explore if the elevated expression of *Insr* and *Akt* in the REG3β$^{-/-}$ mice was mediated by a common transcriptional regulation, we performed a database search by TESS (http://www.cbil.upenn.edu/tess/) (Schug, 2008) with the 5' flanking region of the *Insr, Akt1 and Akt2* gene promoters (~1kb upstream of the transcription starting site) and identified 9 common transcriptional factors (TF) that could potentially bind the proximate promoter of *Insr*, *Akt1*, and *Akt2* (*Akt3* was excluded for little involvement in insulin sensitivity). Although 5 of these TFs [Ccaat-enhancer-binding proteins beta (*C/ebpb,* 45%), *Nr3c1* (47%), hepatocyte nuclear factor-3 beta (*Hnf3b,* 2.7-fold), wilms tumor protein (*Wt1,* 40%) and Ccaat-enhancer-binding proteins delta (*C/ebpd,* 80%)] showed genotype differences ($P < 0.05$) in the hepatic mRNA abundance (Fig. 3.21), only the change of glucocorticoid receptor *Nr3c1* mRNA was reversed (18%, $P < 0.05$) by the 20ng/ml REG3β treatment in the hepatocytes of REG3β$^{-/-}$mice (Fig. 3.22). Therefore, we chose NR3C1 as the most viable candidate for mediating the impacts of REG3β on *Insr* and *Akt1,2*. To map the binding sites of NR3C1 in their promoters, we constructed a series of luciferase reporter plasmids with DNA fragments of *Akt1* and 2 (-1,000 to 0 bp) and *Insr* (-1100 to 0 bp) genes and transfected them into HepG2 cells. Based on their responses to the transcriptional inhibition by 12 hr 20ng/ml REG3β treatment, we narrowed down the binding regions in the *Akt1* and *2* promoters to -250 to 0 bp (up to 90% inhibition, $P < 0.05$) and *Insr* promoter to -1100 to -500 bp (71% inhibition, P <

0.05) (Fig. 3.23). Subsequent ChIP assays to test an actual binding of Nr3c1 to the *Insr/Akt1/Akt2* proximal promoter with designed three or four sets of primers that spanned the 5'-flanking region of *Insr/Akt1/Akt2* (-1,100/-1000 to 0 bp) (*Appendix,* Table 1) and isolated chromatin from WT and KO hepatocytes revealed the actual binding in the proximate promoter of *Insr* at -1100/-761 bp, *Akt1* at -760/-521 and -280/-1 bp, and *Akt2* at -280/-1 bp (dotted circle) (Fig. 3.24). Knocked down (80%, $P < 0.05$) of *Nr3c1* enhanced ($P < 0.05$) mRNA levels of *Insr* (24%), *Akt1* (84%), and *Akt2* (59%) (Fig. 3.25). These enhancements in HepG2 cells were similar to the knockout effects of REG3β on the expression of these genes in the liver of mice, which illustrated importance of the tested NR3C1 binding in the proximate promoters of the three genes in mediating the inhibition of their transcription by REG3β.

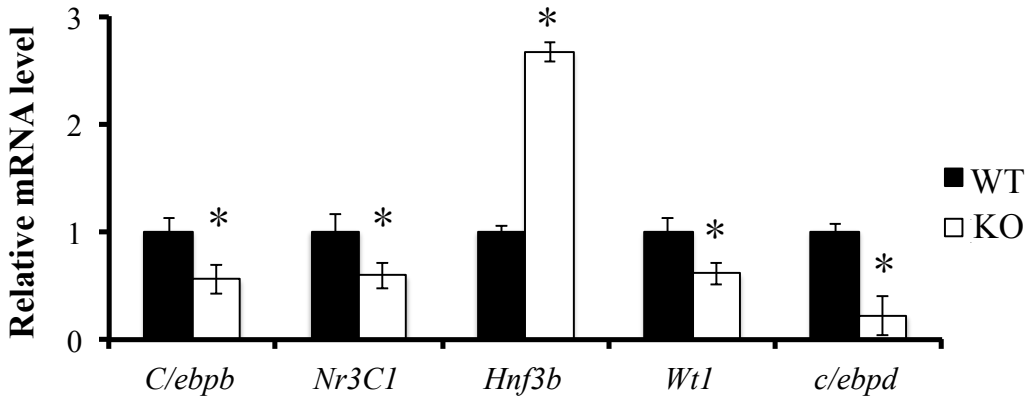

Figure 3.21 Relative mRNA levels of 5 common transcription factors that potentially bind the proximate promoters of *Insr* and *Akt1,2* in the liver of WT and KO mice. Data are mean ± SE (n = 6-7) and normalized to the *β-actin* mRNA levels, *, $P < 0.05$ vs. WT.

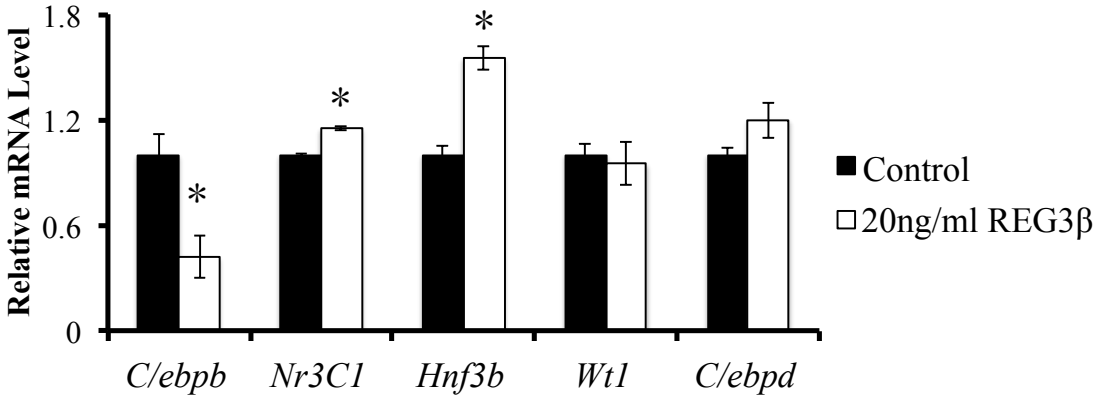

Figure 3.22 Reversing mRNA levels of *Nr3c1* by treating with REG3β (20 ng/mL) for 6 h in primary hepatocytes of the KO mice. Data are mean ± SE (n = 5) and normalized to the *β-actin* mRNA level, *, $P < 0.05$ vs. control.

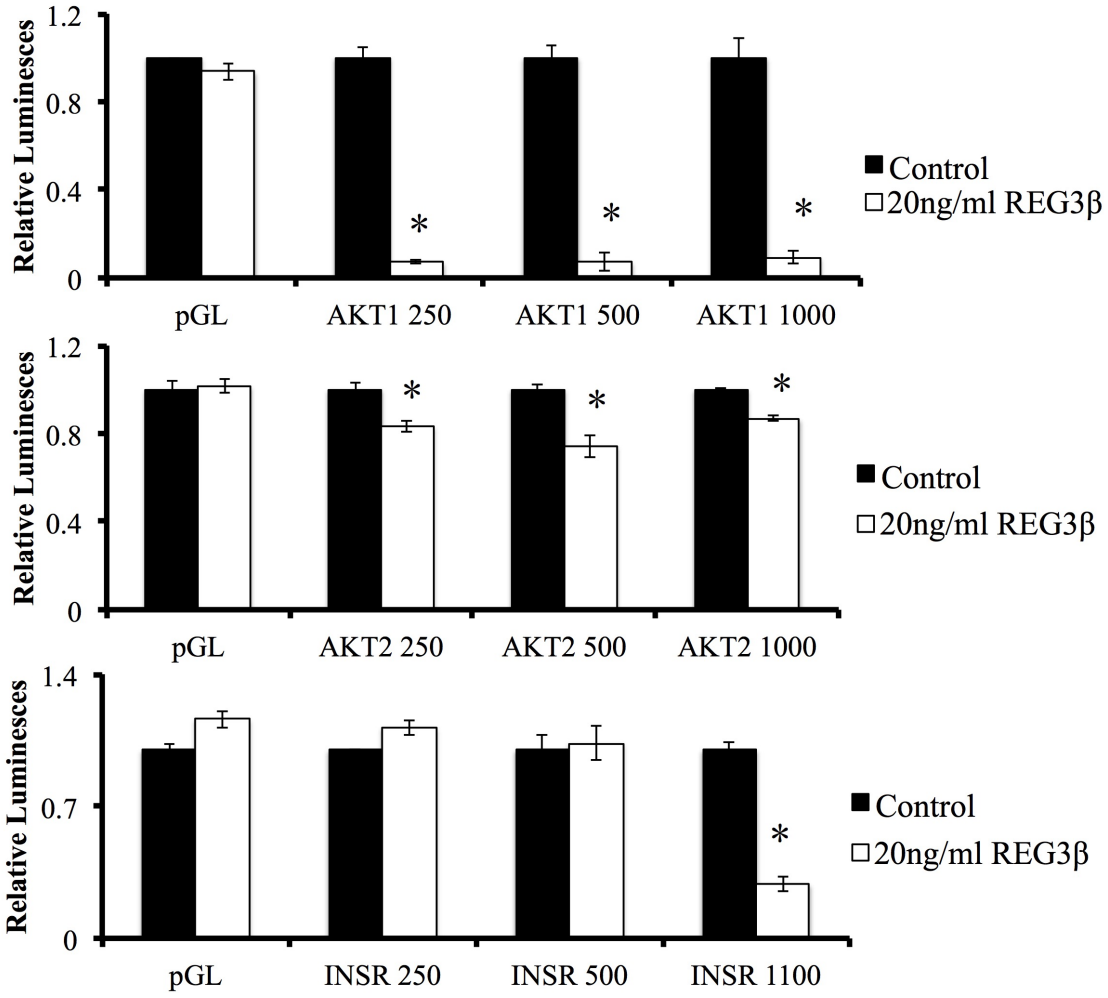

Figure 3.23 Suppressed transcription activity of the promoter of *Insr* (-500bp to -1100bp) (upper panel) and *Akt1* (middle panel) and *2* (0bp to -250bp) (lower panel) in the HepG2 cells transfected with luciferase vector containing different length promoter region of *Insr*, *Akt1* and *Akt2* for 48 h by treating with REG3β (20 ng/mL) for 8 h. Data are mean ± SE (n = 5), and *, P < 0.05 vs. control. Also refer to Figure 3.24

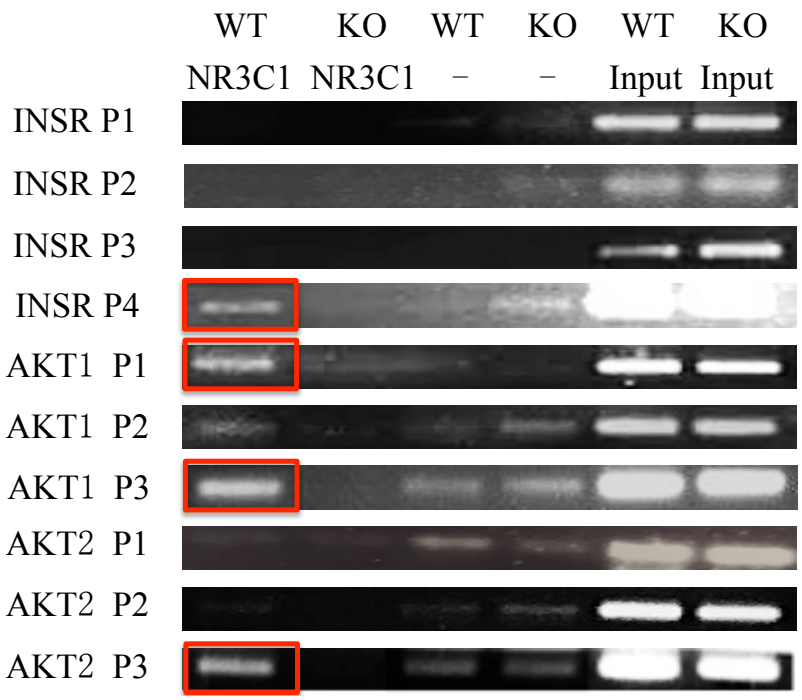

Figure 3.24 Stimulated binding of NR3C1 to the proximate promoters of *Insr*, *Akt1* and *Akt2* by REG3β (P1-P4 represent primer pairs cover different sequence of promoters of *Insr*, *Akt1,* and *Akt2*) as shown by the stronger signal on the left of the fourth [primer set 4 of *Insr* (-1100/-761)], fifth [primer set 1 of *Akt*1 (-760/-521)], seventh [primer 3 (-280/-1) of *Akt*1], and last boxes [primer set 3 of *Akt*2 (-280/-1)] in frame). Chromatin was isolated from the hepatocytes of WT and KO mice after fixation with 4% paraformaldehyde, and pulled down with an anti-NR3C1 antibody. The precipitate was analyzed by PCR. The binding affinity was shown by the intensity of the amplified PCR band signal. The image was a representation of 3 independent assays. Also refer to Figure 3.23.

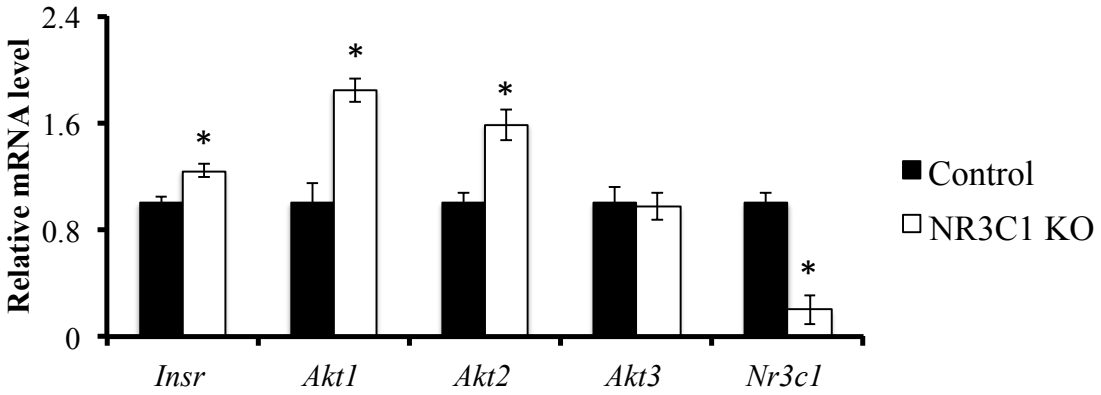

Figure 3.25 Elevated mRNA levels of *Insr* and *Akt1* and *2* by knocking out of *Nc3r1* in the HepG2 cells. Data are mean ± SE (n = 5) and normalized to the *β-actin* mRNA level, *, $P < 0.05$ vs. control.

To link the endocrine-like, extracellular REG3β to the action of NR3C1 in the transcriptional inhibition of *Insr* and *Akt1,2*, we conducted an exhaustive literature search and found EXTL3 (a glycosyltransferase that regulates the biosynthesis of heparan sulfate) (Busse et al., 2007) as the most promising candidate. This was because: 1) EXTL3 is be able to bind the human homologue of REG3β: hREG3α and REG1α (Van Ba et al., 2012; Wu et al., 2016b); and 2) heparan sulfate proteins, produced by the catalysis of EXTL3 (Busse et al., 2007), could activate NR3C1 via stepwise interactions with fibroblast growth factor and fibroblast growth factor receptor complex to activate ERK1/2 (Galanternik et al., 2015), the complex of tau 1 transactivation domain of NR3C1(Dahlmanwright et al., 1994) with cAMP response element binding protein (Almlof et al., 1998). To test whether REG3β bound EXTL3, we overexpressed EXTL3 in HepG2 cells (Fig. 3.26) and showed a co-IP of the two proteins by incubating the homogenates of HepG2 cells with or without EXTL3 with native REG3β produced in *P. pastoris* (*Appendix,* Table 3). The bound complex was pulled down using an EXTL3 antibody and the physical binding between these two proteins was verified by immunoblotting the precipitates against a REG3β antibody (Fig. 3.27). Their interaction was confirmed by the consistent result of dual-fluorescent co-localization in the cells as we generated native REG3β-EGFP in HEK293T cells (EGFP protein was used as a control) and then added these exogenous proteins into the culture media of HepG2 cells overexpressing EXTL3-mCherry. After the incubation, cells were washed and observed under microscope. Consistently, only the REG3β-EGFP treatment yielded yellow fluorescence, which is resulted from an overlap of the green (REG3β-EGFP) and the red fluorescence (EXTL3-mCherry) on the cell surface. This effect was not seen with the

EGFP control. Furthermore, this binding could be inhibited by the addition of EXTL3 antibody into the reaction (Fig. 3.28). Thereafter, we performed a knockdown of *Extl3* in the HepG2 cells that duplicated the impacts of REG3β knockout in the tissues: 19-79% upregulation of *Insr* and *Akt1,2* mRNA, and decreased heparan sulfate proteins production (Fig 3.29 and 3.30). More directly, knockdown of *Extl3* totally blocked the effects of exogenous REG3β on *Insr*, *Akt1,2*, and *Nr3c1* (Fig 3.32). Altogether, we proved the binding of REG3β to EXTL3 to initiate the transcriptional inhibition of *Insr* and *Akt1,2*.

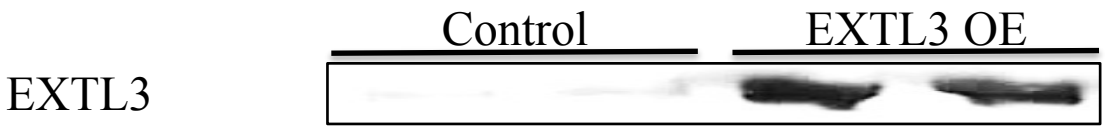

Figure 3.26 Overexpressed EXTL3 protein in the HepG2 cells, as shown by immunoblotting. Image was a representation of 3 independent experiments. Also refer to Figure 3.27.

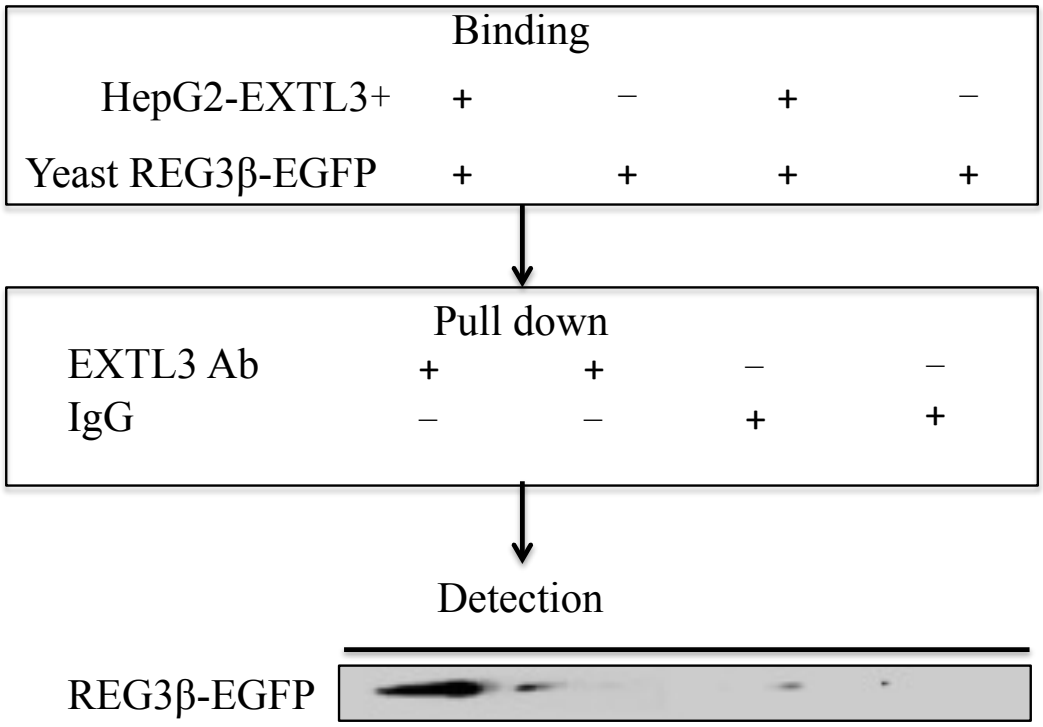

Figure 3.27 A specific co-immunoprecipitation of REG3β and EXTL3 proteins. After EXTL3 was overexpressed in the HepG2 cells for 48 h, the homogenate was incubated with the native REG3β-EGFP protein produced in yeast. The binding complex was precipitated with the anti-EXTL3 antibody, and the precipitate was immunoblotted against anti-REG3β antibody. The image was a representation of 3 independent assays. Also refer to Figure 3. 26.

EGFP	–	+	–	–	Treatment
REG3β−EGFP	+	–			
HepG2-mCherry+	+	–	–	–	Overexpression
HepG2−mCherry-EXTL3+	–	+	+	+	
EXTL3 Ab	–	–	–		Blocking

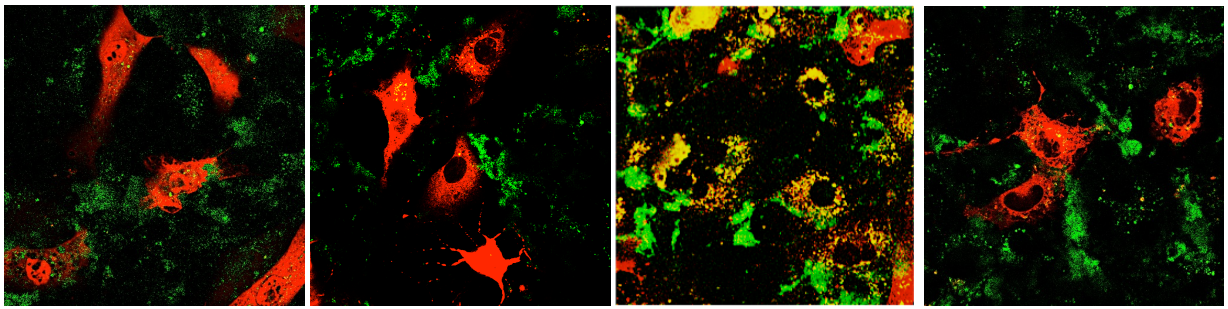

Figure 3.28 Binding of exogenous REG3β-EGFP (green fluorescence) overexpressed in HEK293 T cells and added in the media to EXTL3-mCherry (red fluorescence) overexpressed in HepG2 cells for 48 h on the cell surface yielded yellow fluorescence (the 3rd box), resulting from the co-localization of the two proteins. This binding was not induced by either adding EGFP or in cells overexpressing mCherry, but was blocked by the EXTL3 antibody added in the media (the 4th box). The image was a representation of 3 independent assays.

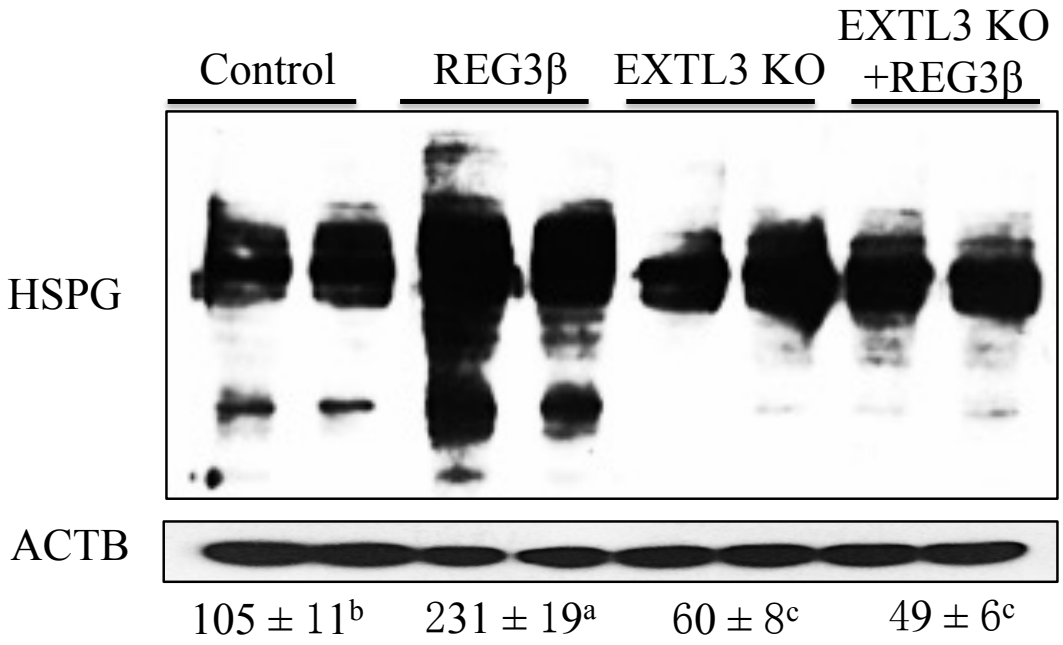

Figure 3.29 Blocked responses of heparan sulfate proteoglycans on the cells surface to the treatment of REG3β (20 ng/mL for 6 h) in the HepG2 cells by the knock down of EXTL3. Data are mean ± SE (*n* = 5), and data with different letters differ. Also refer to Figure 3.30.

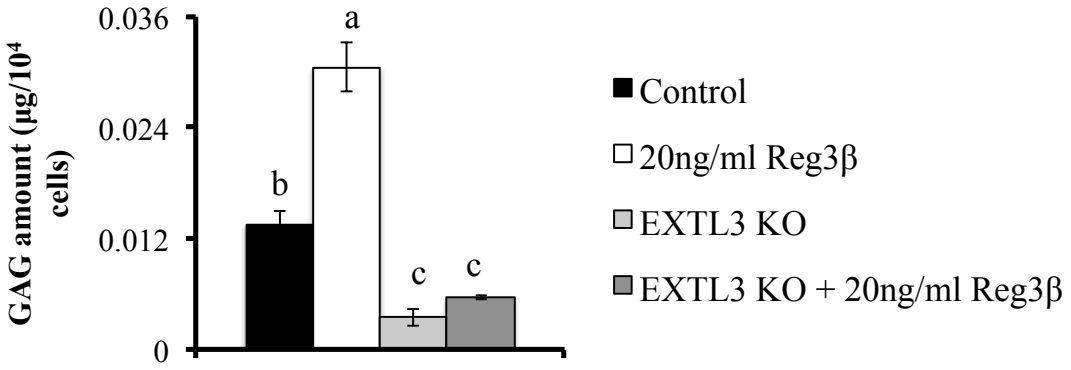

Figure 3.30 Blocked responses of GAG chains on the cells surface to the treatment of REG3β (20 ng/mL for 6 h) in the HepG2 cells by the knock down of EXTL3. Data are mean ± SE (n = 5), and data with different letters differ. Also refer to Figure 3.29 and 3.32.

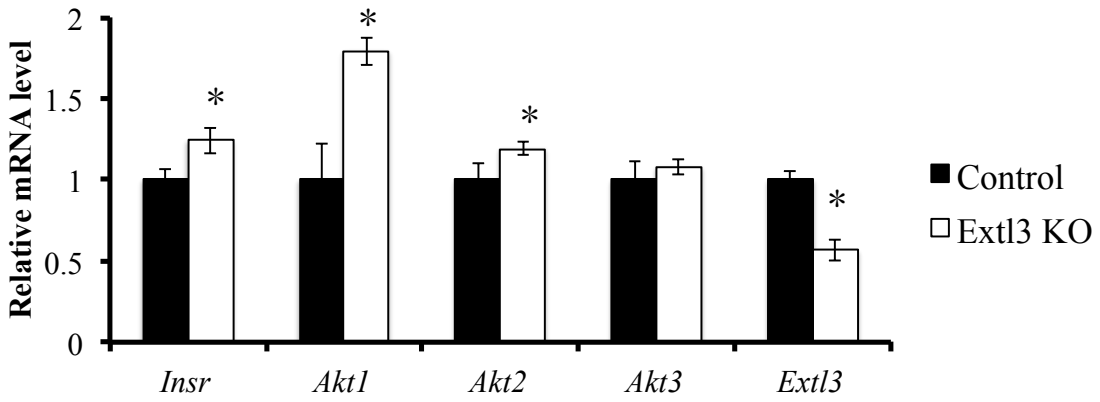

Figure 3.31 Elevated mRNA levels of *Insr* and *Akt1-2* in the HepG2 cells by knocking down EXTL3. Data are mean ± SE (n = 5) and normalized to the *β-actin* gene mRNA level. *, $P < 0.05$ vs. control. Also refer to Figure 3. 32.

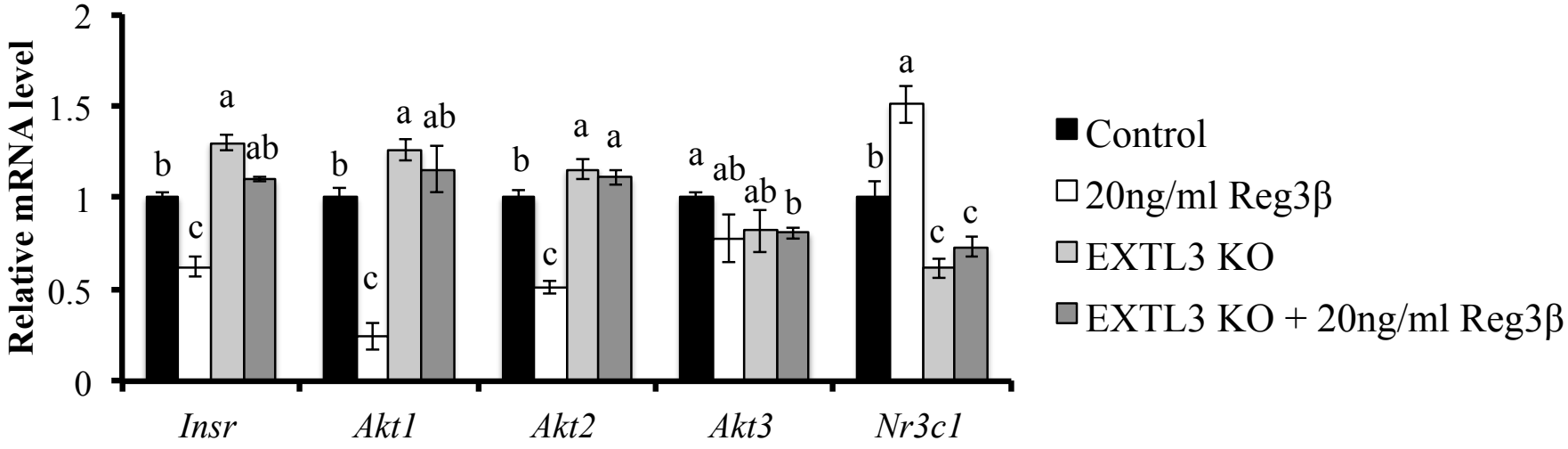

Figure 3.32 Blocked responses of *Insr, Akt1-2* and *Nr3c1* mRNA levels to the treatment of REG3β (20 ng/mL for 6 h) in the HepG2 cells by the knockout of EXTL3. Data are mean ± SE (n = 5) and normalized to the *β-actin* mRNA levels. Data with different letters differ ($P < 0.05$). Also refer to Figure 3.30 and 3.31.

3.4 The binding of REG3β to transmembrane CXCR4 activated SOCS3, leading to subsequent inhibition of INSR and AKT phosphorylation

To reveal mechanisms for the ablated IR induced by LPS and DSS-mediated inflammation in the REG3β$^{-/-}$ mice, we determined effects of these compounds on insulin-stimulated phosphorylation of tissue INSR and AKT. While LPS abolished or decreased (47% to 100%, $P < 0.01$) P-INSR and(or) P-AKT in the liver and muscle of WT, it affected little or even enhanced their phosphorylation in these tissues of REG3β$^{-/-}$ mice (Fig. 3.33). As P-INSR and P-AKT in inflammation are largely decreased by SOCS3 (Nieto-Vazquez et al., 2008), we followed up its responses to the LPS treatment. While hepatic SOCS3 was enhanced ($P < 0.05$, 42%) in the WT by LPS, no change was seen in the REG3β$^{-/-}$ mice (Fig. 3.34). Comparatively, responses of c-Jun N-terminal kinase (P-JNK) and P38 MAP kinase (P38) that were also reported to affect the phosphorylation of INSR and AKT (Nieto-Vazquez et al., 2008) were mixed (Fig. 3.34). Because SOCS3 was shown to be directly stimulated by CXCR4 (Takekoshi et al., 2013), a well-known transmembrane inflammatory protein (Wescott et al., 2016) that was induced by LPS (Fig. 3.34), in the presence of its ligand CXCL12, and the CXCL12/CXCR4 complex was speculated as the target of REG3β in cancer cells (Loncle et al., 2015), we hypothesized that lack of this putative binding might block the inflammation-induced IR in the REG3β$^{-/-}$ mice.

We performed cross-linking co-IP using extracellular REG3β-EGFP overproduced in HepG2 cells to bind the intrinsic CXCR4 protein on the surface of the HepG2 cells in the presence of added CXCL12-His overexpressed in *P. pastoris* (*Appendix,* Table 3) in the

media compared with non-overproduced REG3β-EGFP cells. After the target binding, the complex was cross-linked with 1mM disuccinimidyl suberate and the complex were pulled down by the EGFP affinity beads, the physical interactions among these three proteins were verified by immunoblotting using three antibodies against REG3β, CXCR4, and CXCL12-His-tag, respectively (*Appendix* Table 3, Fig. 3.35). This binding was clearly dependent on the level of REG3β-EGFP in the media and was only seen when both the REG3β-EGFP protein and EGFP affinity beads were added into the mixture. In addition, the putative binding between the overexpressed mCherry-CXCR4 in HepG2 cells with the added REG3β-EGFP overexpressed in HEK293 T cells in the media was confirmed by the consistent result of the dual-fluorescent co-localization assay, as only the REG3β-EGFP treatment yielded yellow fluorescence resulted from an overlap of the green (REG3β-EGFP) and the red fluorescence (CXCR4-mCherry) on the cell surface, whereas the EGFP or mCherry protein itself did not show any positive signal and this binding was blocked by CXCR4 antibody (Fig. 3.36 and 3.37). Specificity of this binding was shown by the negative outcome with another inflammation-related protein GP130 that was also suggested to bind to REG3β (Loncle et al., 2015) (Fig. 3.38). Therefore, we proved our hypothesis that the inflammation-elevated REG3β could bind CXCL12/CXCR4 complex to stimulate SOCS3, inhibiting phosphorylation of INSR and AKT.

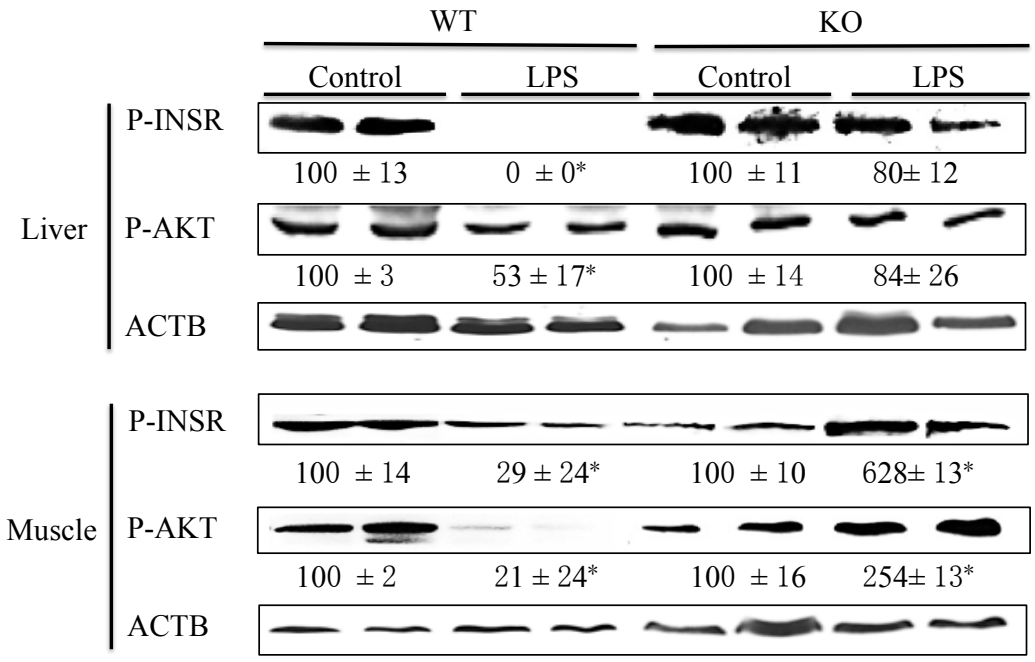

Figure 3.33 Abolished or decreased insulin-stimulated phosphorylation of INSR and AKT protein at 12 h after the injection of LPS (2 mg/kg), shown by immunoblotting, in the liver (upper panel) and muscle (lower panel) of WT mice but not in the KO mice (2-month old). Data are mean ± SE (n = 5) and normalized to β -Actin as the loading control, *, $P < 0.05$ vs. WT/KO control.

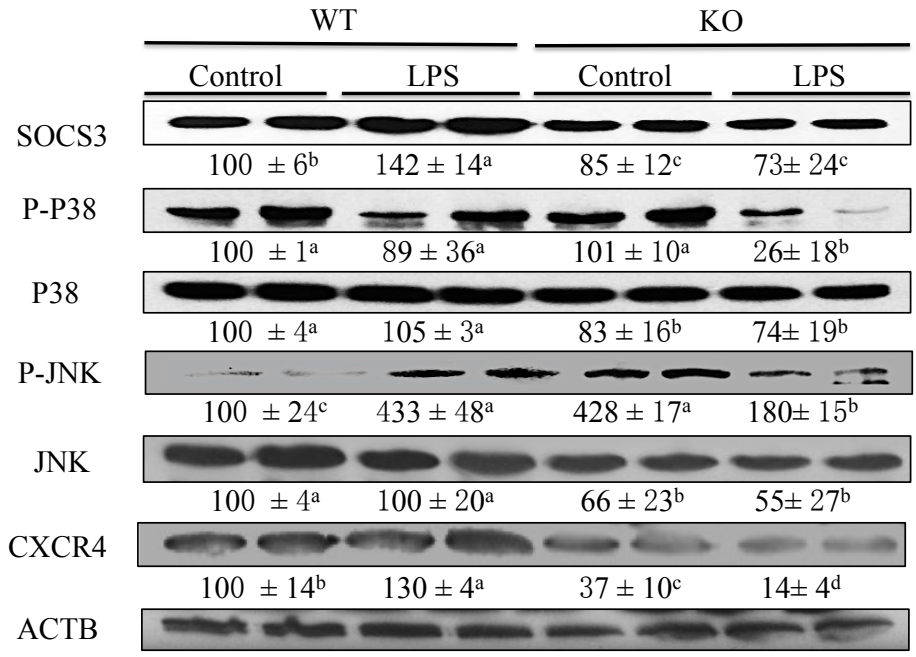

Figure 3.34 Elevation of SOCS3 and CXCR4 and mixed responses of other 4 inflammation-IR-related factors in amounts of phosphorylated or total proteins at 12 h after the injection of LPS (2 mg/kg), shown by immunoblotting, in the liver of the WT, but not in the KO mice. Data are mean ± SE (n = 6) and normalized to β -ACTIN as the loading control. Data with different letters differ, $P < 0.05$.

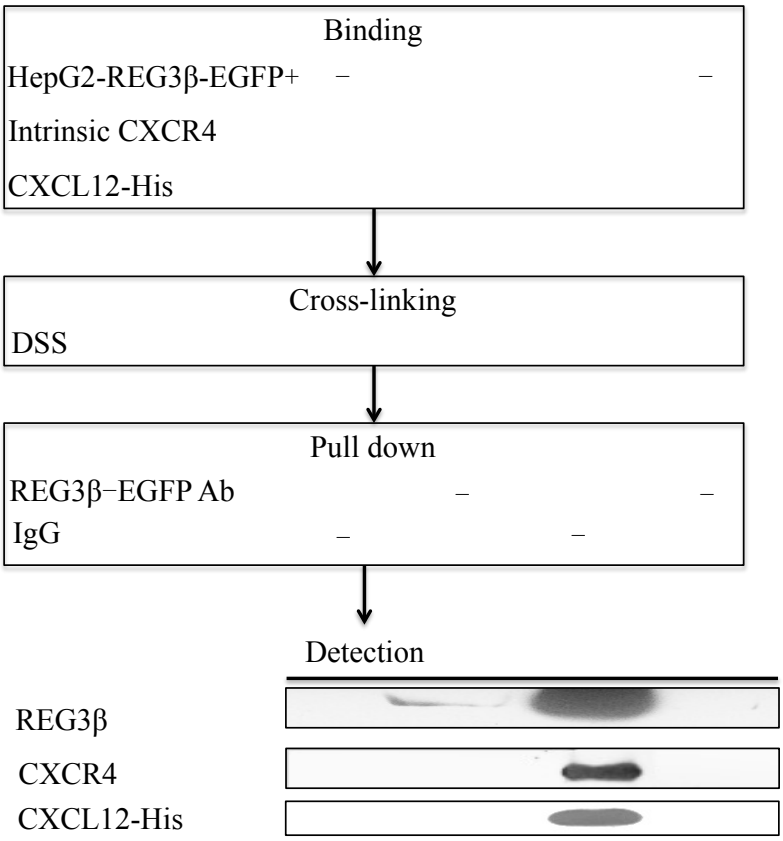

Figure 3.35 A specific co-immunoprecipitation of REG3β, CXCR4, and CXCL12 proteins. After REG3β-EGFP was overexpressed extracellularly in the HepG2 cells for 48 h and bound with intrinsic CXCR4 in the presence of CXCL12-His (produced in yeast) added into the medium, the binding complex was cross-linked by 1 mM disuccinimidyl suberate (DSS) for 30 min. The cells were homogenized and the binding complex was precipitated with EGFP affinity beads. The precipitate was immunoblotted against anti-CXCR4, His, and REG3β antibodies. The image was a representation of 3 independent assays. Also refer to Figure 3.38.

Zeping Zhao

EGFP	–	+	–	–	Treatment
REG3β−EGFP	+	–			
HepG2-mCherry	+	–	–	–	Overexpression
HePG2-mCherry-CXCR4	–	+	+	+	
CXCR4 Ab	–	–	–		Blocking

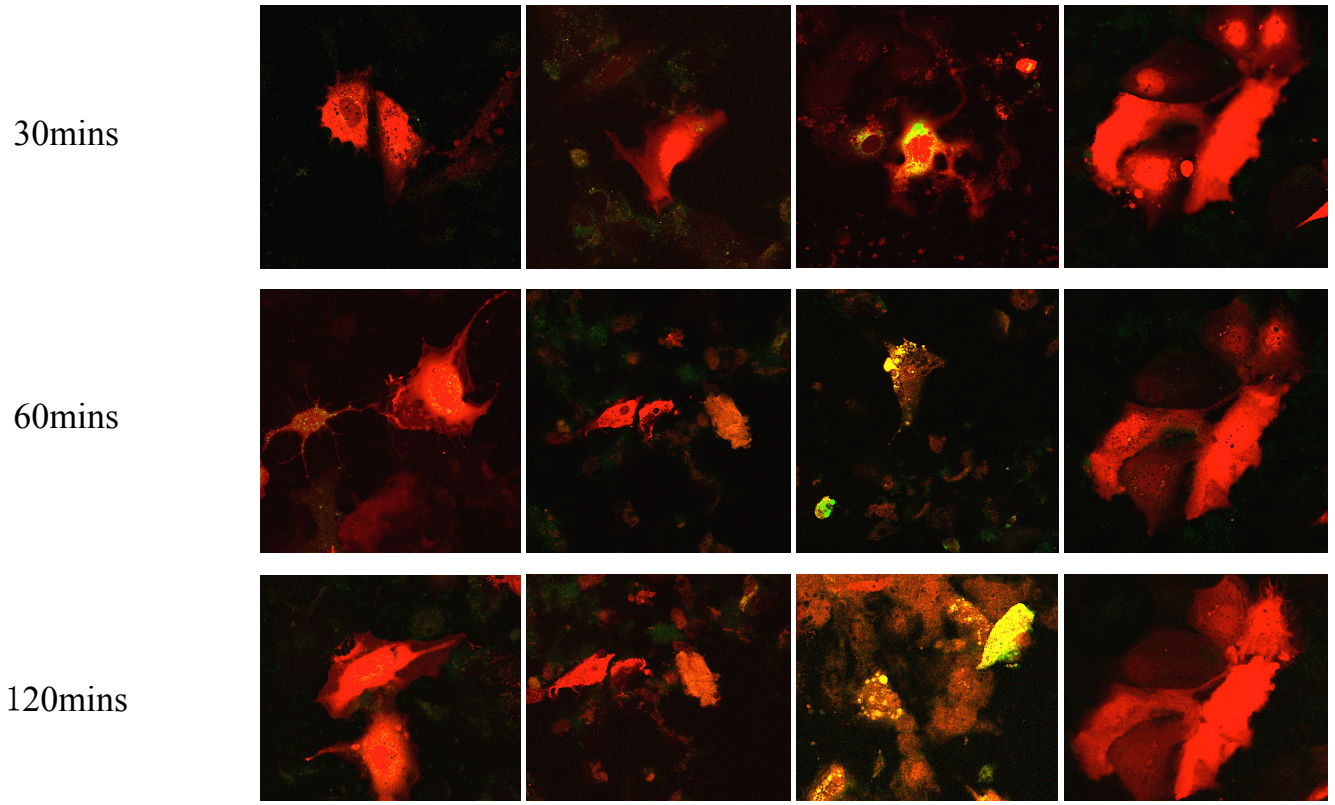

Figure 3.36 Binding of added REG3β-EGFP (green fluorescence) overexpressed in HEK293 T cells in the media to CXCR4-mCherry (red fluorescence) overexpressed in HepG2 cells for 48 h on the cell surface, as shown by the yellow fluorescence (the 3rd panel) resultant from the co-localization of REG3β-EGFP and CXCR4-mCherry. The binding was not induced by either EGFP or in cells overexpressing mCherry, but was blocked by the CXCR4 antibody in the media (the 4th panel). The image was a representation of 3 independent assays. Also refer to Figure 3.38

EGFP		+			Treatment
REG3β−EGFP	+		+	+	
HepG2-mCherry+	+				Overexpression
HepG2-mCherry-CXCR4+		+	+	+	
CXCR4 Ab				+	Blocking

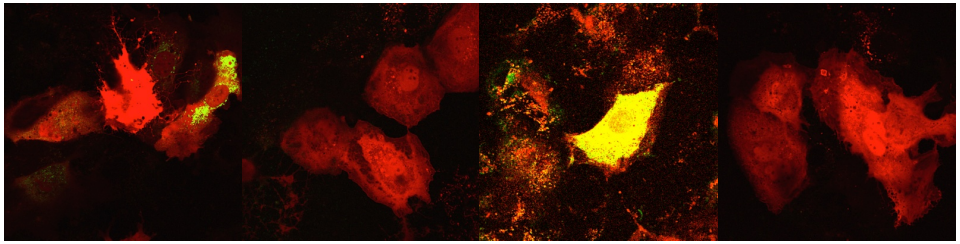

Figure 3.37 Binding of added REG3β-EGFP (green fluorescence) overexpressed in HEK293 T cells in the media to CXCR4-mCherry (red fluorescence) overexpressed in HepG2 cells for 48 h on the cell surface, as shown by the yellow fluorescence (the 3rd panel) resultant from the co-localization of REG3β-EGFP and CXCR4-mCherry. The binding was not induced by either EGFP or in cells overexpressing mCherry, but was blocked by the CXCR4 antibody in the media (the 4th panel). The image was taken at 2 h after the binding was induced and was a representation of 3 independent assays. Also refer to Figure 3.36.

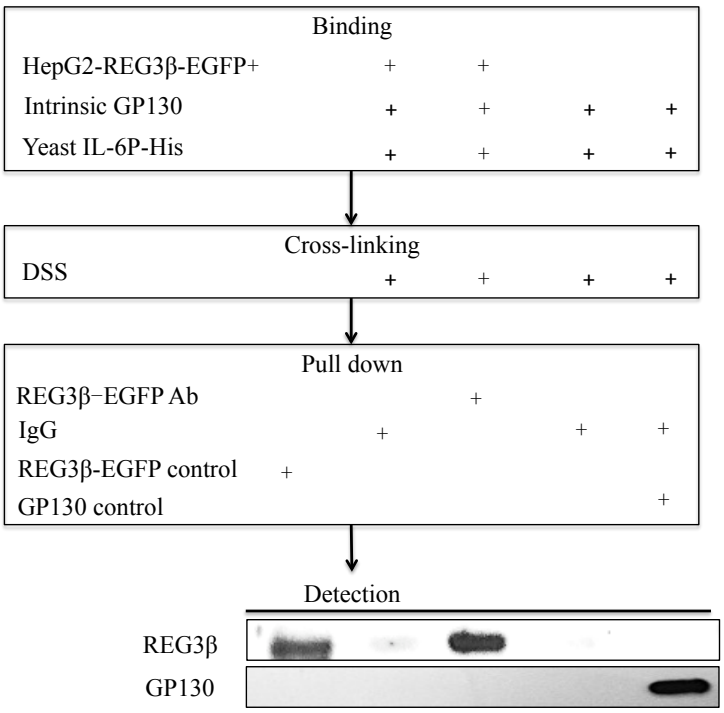

Figure 3.38 Failure to precipitate GP130 by REG3β proteins. After REG3β-EGFP was overexpressed extracellularly in the HepG2 cells for 48 h and incubated with intrinsic GP130 in the presence of IL-6-His (produced in yeast) added into the medium, the intended binding complex was cross-linked by 1 mM disuccinimidyl suberate (DSS) for 30 min. The cells were homogenized and the binding complex was precipitated with EGFP affinity beads. The precipitate was immunoblotted against anti-GP130, His, and REG3β antibodies. The image was a representation of 3 independent assays. Also refer to Figure S3C. Also refer to Figure 3.33 and 3.35.

3.5 Pre-clinical testing of manipulating REG3β and its human homolog hREG3α on insulin sensitivity and effects of 5 commonly-used anti-inflammatory drugs on tissue REG3β protein production

To explore the physiological relevance and clinical potential of improving insulin sensitivity by removing REG3β, we injected (ip) its antibody into mice with a strong IR (McClung et al., 2004). Compared with the vehicle control, the antibody injections (every other day for 2 wk) improved (16%, $P < 0.05$) insulin sensitivity (Fig. 3.39) and 17% decreased fasting blood glucose (Fig. 3.40), without affecting body weights (Fig. 3.41) compared with the PBS-injected control. While REG3α is considered the human homolog of murine REG3β in humans, to our best knowledge, solid proof is still lacking. Therefore, to test the potential of identifying REG3α as the target in human to improve insulin sensitivity, we inject (i.p.) both WT and REG3β$^{-/-}$ mice with hREG3α, 15 min prior to ITT. In contrast to removing REG3β, the 4 μg/g of body weight hREG3α injecting, decreased 15% ($P < 0.05$) insulin sensitivity in both genotypes (Fig. 3.42 and Fig. 3.43) than the PBS-injected mice. This illustrated similar functions and the clinical potential of neutralizing both REG3β and hREG3α for improving insulin sensitivity. To test if responses of REG3β could help explain the conflicting effects of anti-inflammatory drugs on IR (Buren et al., 2002; da Silva et al., 2018; Ford et al., 2015), we treated the WT with 5 commonly-used AIT drugs after the LPS challenge (Buren et al., 2002; da Silva et al., 2018; Ford et al., 2015). Actual effects of these 5 drugs on IR were remarkably consistent with their ability to regulate REG3β protein (Fig. 3.44): 0.12 mg/kg body weight 6 hr treatment of DEX aggravates IR elevated the protein in pancreas

($P < 0.05$); 5 mg/kg bodyweight 2 wk treatment of sulindac and 40mg/kg body weight 1 hr treatment of statin do not affect IR failed to change the protein expression induced by 2mg/kg body weight LPS treatment; and 81mg/kg body weight 1 hr treatment of aspirin and 40mg/kg body weight 2wk treatment of ibuprofen that alleviate IR decreased the protein expression induced by 2mg/kg body weight LPS treatment.

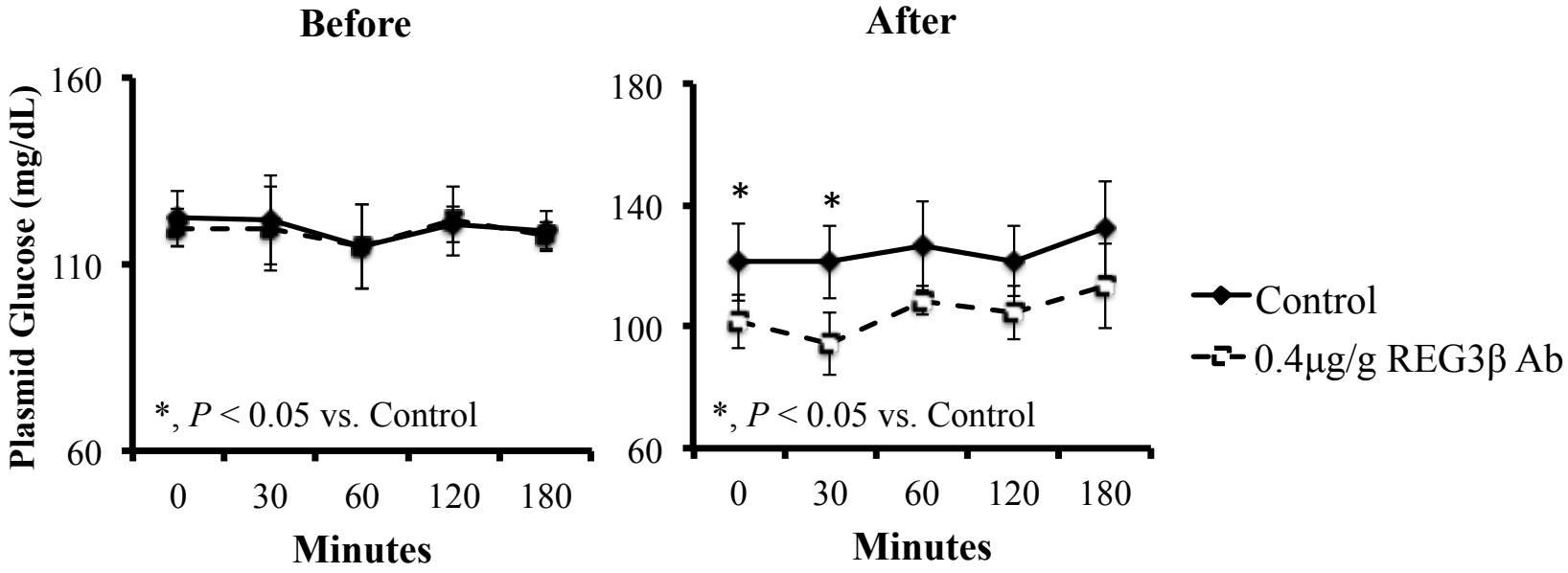

Figure 3.39 Enhanced insulin sensitivity, as shown by ITT (0.5 U/kg), in the insulin resistant mice by i.p. injections of anti-REG3β antibody (0.4 μg/g, every other day for 2 wk) compared with the pre-immune serum-injected control mice. Data are mean ± SE (n = 10), and *, $P < 0.05$ vs pre-immune serum control. Also refer to Figure 3.40 and 3.41.

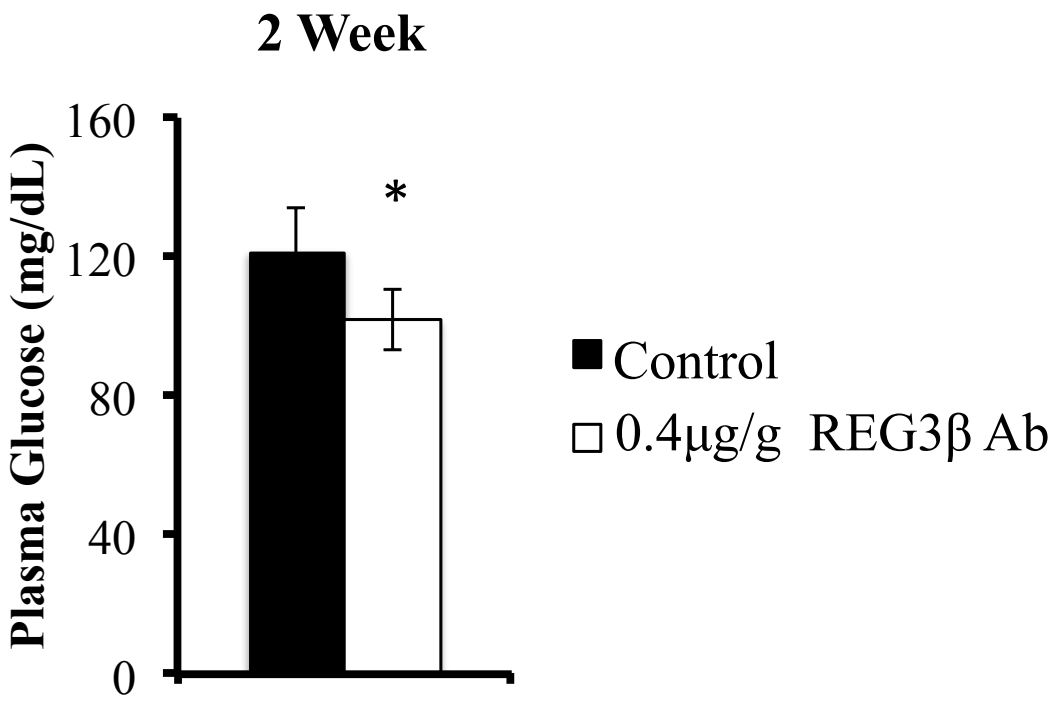

Figure 3.40 Decreased plasma glucose in the insulin resistant mice by i.p. injections of anti-REG3β antibody (0.4 μg/g BW, every other day for 2 wk) compared with the pre-immune serum-injected control mice after 8 h overnight fasting. Data are mean ± SE (n = 10), and *, $P < 0.05$ vs pre-immune serum controls. Also refer to Figure 3.39.

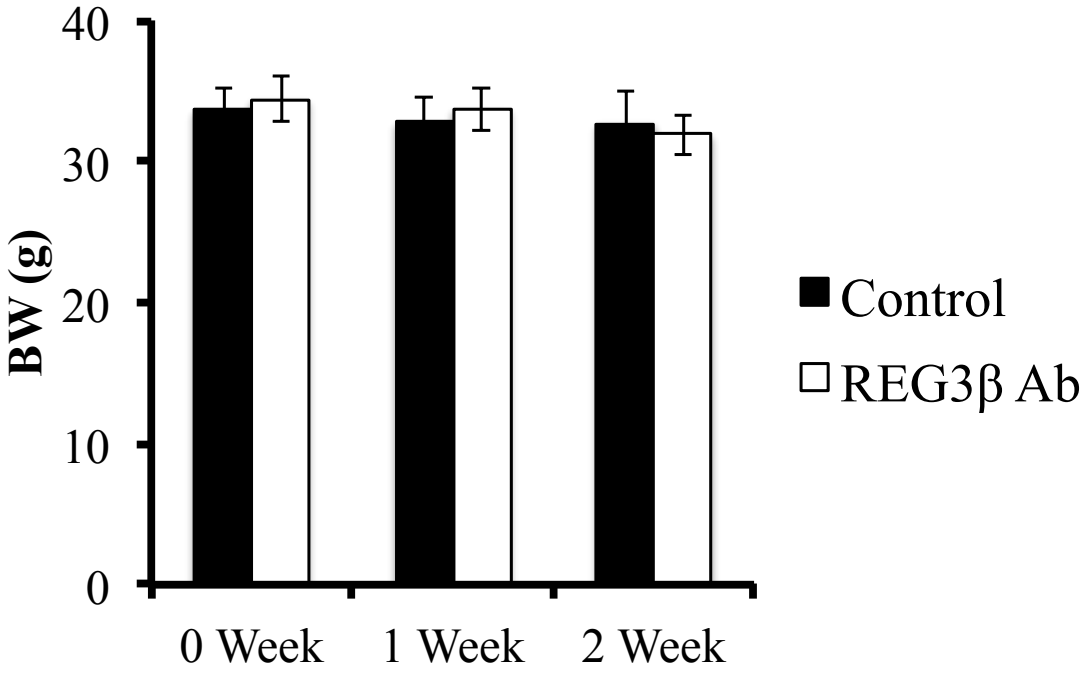

Figure 3.41 Lack of difference in body weight in the insulin resistant mice by i.p. injections of anti-REG3β antibody (0.4 μg/g BW, every other day for 2 wk) compared with the pre-immune serum-injected control mice after 8 h overnight fasting. Data are mean ± SE (n = 5), and *, $P < 0.05$ vs. pre-immune serum controls. Also refer to Figure 3.39.

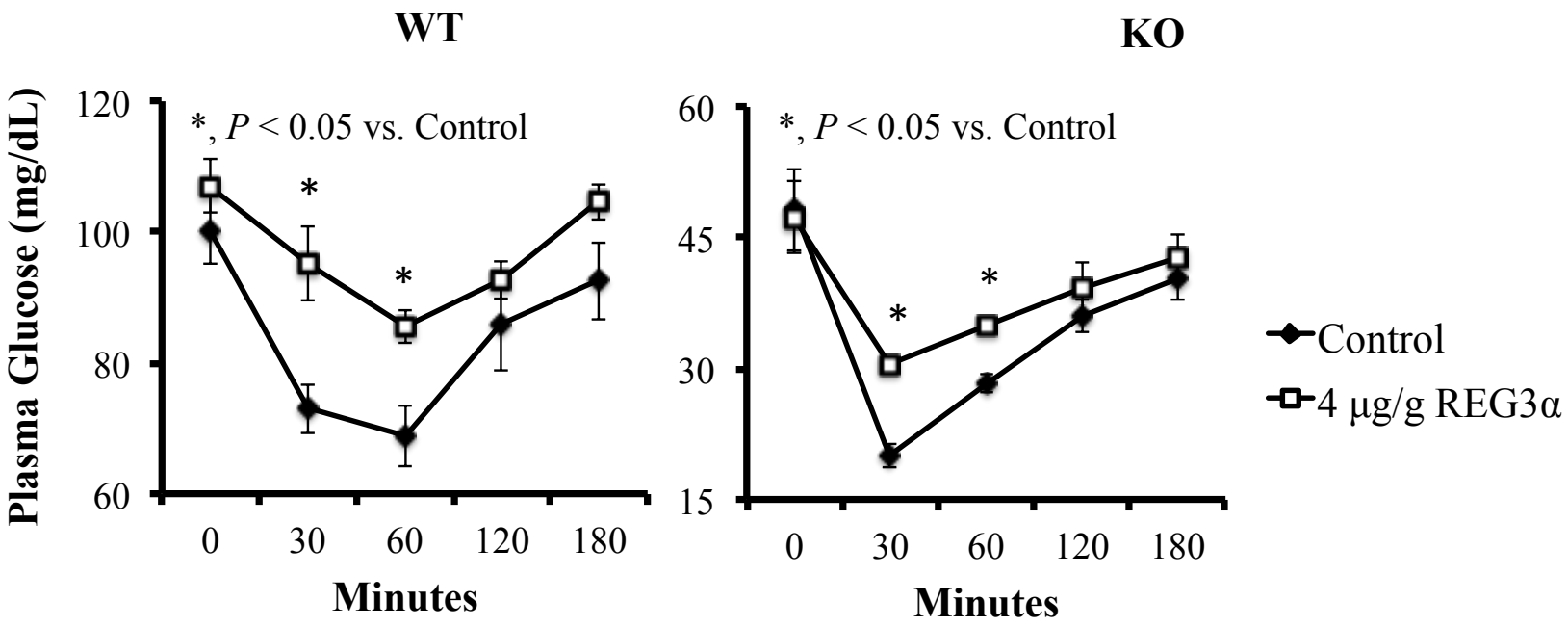

Figure 3.42 Decreased insulin sensitivity, as shown by ITT (0.5 U/kg), in the WT and KO mice by a single i.p. injection of the native REG3α (4 μg/g body weight) at 15 min prior to ITT, compared with the PBS-injected controls. Data are mean ± SE (n = 10), and *, P < 0.05 vs the PBS controls. Also refer to Figure 3.43.

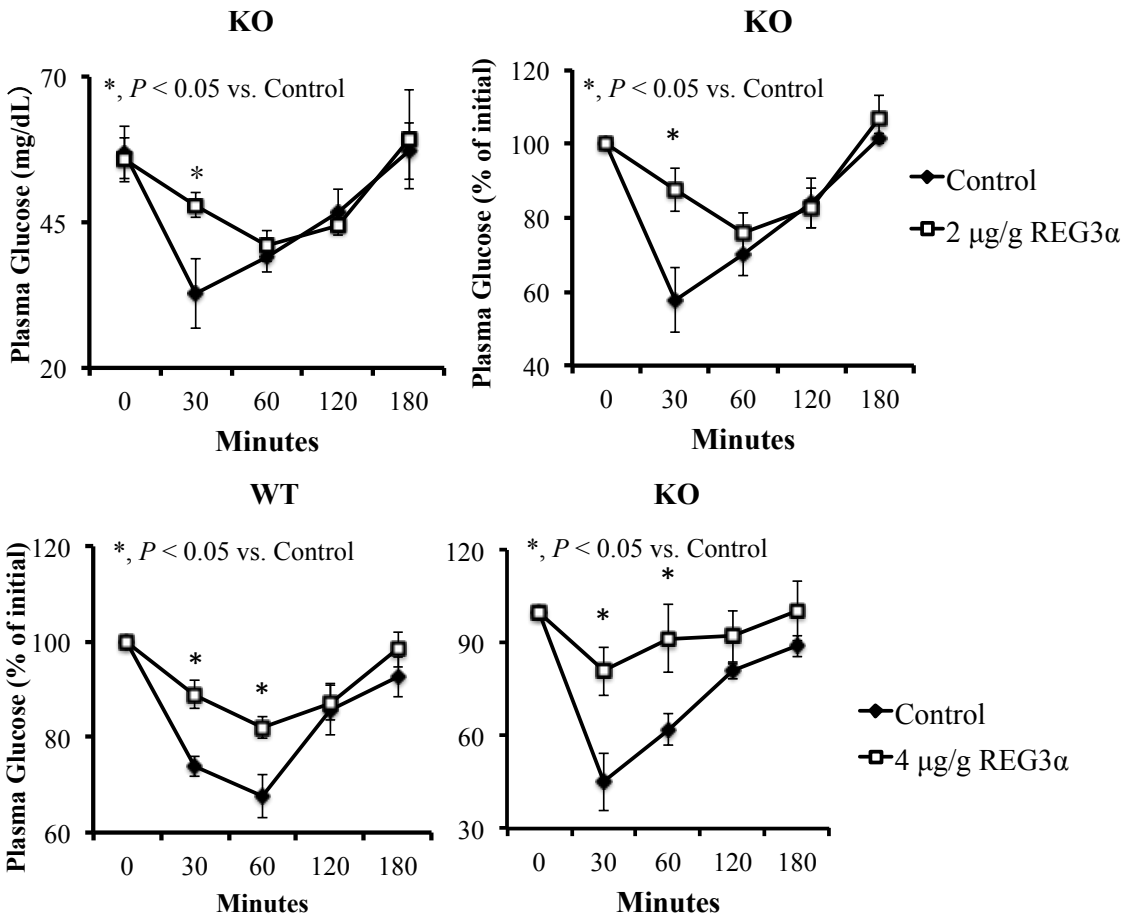

Figure 3.43 Decreased insulin sensitivity, as shown by ITT (0.5 U/kg), in the WT and KO mice by a single i.p. injection of the native REG3α (2 or 4 μg/g BW) at 15 min prior to ITT, compared with the PBS-injected controls. Data are mean ± SE (n = 10), and *, P < 0.05 vs the PBS controls. Also refer to Figure 3.42.

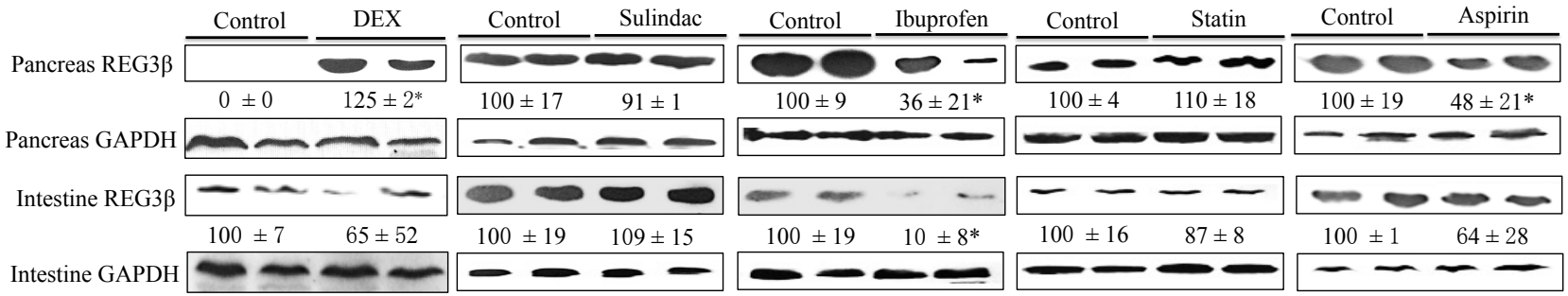

Figure 3.44 Effects of 5 commonly-used anti-inflammatory drugs on the REG3β protein production, shown by immunoblotting, in the pancreas and intestine of WT mice. Data are mean ± SE (n = 6) and normalized to β -ACTIN as the loading control, *, $P < 0.05$ vs. controls.

CHAPTER FOUR

DISCUSSION

4.1 Discussion

Our most exciting discovery is that knockout of REG3β enhanced baseline insulin sensitivity and blocked the inflammation-induced IR in the mice. This finding enables three major conceptual breakthroughs: 1) REG3β acted as a novel, potent, and negative determinant of the baseline insulin sensitivity; 2) REG3β was required for the systemic inflammation to induce IR; and 3) REG3β functioned beyond regeneration and anti-inflammation. These breakthroughs will have significant implications to advance the broad field of biomedical science related to diabetes, inflammation, and regeneration. Although several previous studies implied a potential association of REG3β and hREG3α with IR (Calderari et al., 2014; Wu et al., 2016a; Xiong et al., 2016), our study provides the first direct and two-way (reciprocal) evidences to reveal its potent role in controlling baseline insulin sensitivity, and will enable a completely new therapy prospect and target to improve IR in diabetes. More strikingly, demonstrating the essentiality of REG3β, a recognized anti-inflammatory protein (Aden et al., 2016), for the LPS and DSS-induced IR may drastically revise the current theory on the inflammation-induced IR (Ghorpade et al., 2018; Li et al., 2015; Tian et al., 2016). This indicates that the inflammation-induced IR requires a co-existence or stimulation of anti-inflammatory proteins such as REG3β with the inflammatory cytokines and interleukins protein (Aden et al., 2016; Jorgensen et al., 2013; Kim et al., 2004; Matsubara et al., 2012). Thereby, this type of co-stimulation or existence may help explain why not all types of inflammation necessarily induce IR

(Tian et al., 2016), and why altering only inflammatory factors such as TNFα (Ventre et al., 1997) and IL-1R antagonist (Larsen et al., 2009) fails to improve insulin sensitivity. Conversely, anti-inflammatory proteins such as REG3β may serve as a better target or a simultaneous target with inflammatory factors to treat the inflammation-induced IR. Revealing a new role of REG3β in insulin sensitivity and inflammation-induced IR, in addition to the previously reported functions in tissue regeneration (Lorchner et al., 2015), cancer (Liu et al., 2015) and anti-microbe/inflammation (Aden et al., 2016; Miki et al., 2012), may suggest a unique, fundamental position for this endocrine-like molecule to link or coordinate various metabolic processes.

Our study unveils the binding of REG3β to two transmembrane proteins for exerting its long and short-term effect on the baseline insulin sensitivity and the inflammation-induced IR, respectively (Fig. 7). The long-term effect was conferred by binding to EXTL3 to activate NR3C1 that bound the proximate promoters of *Insr, Akt1,* and *Akt2* to inhibit the transcription of these genes. Our work carefully elucidated how knockout of REG3β removed this inhibition, leading to the elevated transcripts and proteins of INSR and AKT. Because previous research showed only the binding of FOXO1 in the *Insr* promoter in the absence of insulin (Puig and Tjian, 2005). However, FOXO1 was reported had dual functions in promoting insulin sensitivity as well as stimulating hepatic lipid synthesis and hepatic glucose production (Matsumoto et al., 2006; Nakai et al., 2002). Thus, the application of FOXO1 as a promising therapeutic target to treat insulin resistance is limited. Previous research showed that NR3C1 was associated with GSIS (van Raalte et al., 2012) and insulin level (van Rossum et al., 2002). Moreover, the

inhibition of glucocorticoids on insulin sensitization is documented (Geer et al., 2014). Even though ChIP assay identified several target genes of GR including insulin signaling transactivation genes (Wang et al., 2004), binding of GR to the promoter of *Insr/Akt1/Akt2* has never been reported. The binding of NR3C1, activated by the interaction of REG3β and EXTL3, to the proximate promoters of *Insr/Akt1/Akt2*, represents a rather new mechanism for the transcriptional regulation of key insulin signal proteins. This also explains the reported association of NR3C1 and its ligand-glucocorticoids with IR (Kang et al., 2015).

At the same time, the short-term or immediate effect was mediated by binding to CXCR4 to stimulate SOCS3 that subsequently inhibited the insulin-stimulated phosphorylation of INSR and AKT. Because both REG3β and CXCR4 could be induced by glucocorticoids (Luo et al., 2013; Wang et al., 1998) and inflammatory stimulants such as LPS (this study), illustrating the actual binding of CXCR4/CXCL12 to REG3β not only fills the previous knowledge gap (Buren et al., 2002; da Silva et al., 2018; Ford et al., 2015; Larsen et al., 2009; Ventre et al., 1997) but also offers a structure mechanism to link their functions. Previously, GP130 and CXCL12/CXCR4 complex were speculated as the target of REG3β in cancer cells (Loncle et al., 2015) but the supporting binding experimental data were not demonstrated. Our study represents the first attempt to reveal that CXCR4 as the tranmembrane protein and receptor for CXCL12 physically interact with REG3β and no interaction was observed between GP130 and REG3β even though GP130 is proved as a target of REG3β.

Tremendous clinical potential is derived from our research. The potent inhibition of baseline insulin sensitivity by REG3β, the improved insulin sensitivity by its antibody in the IR mice, and the similar function of the human homologue hREG3α consistently point out not only the confirmation of homologues between REG3β and hREG3α but also REG3β/hREG3α as a powerful, new therapy target to treat IR in diabetes as many beneficial drugs were limited by severe side effects including nausea (Fujii et al., 2012), vomiting (Merrill et al., 2010), risk of organ bleeding (Garcia Rodriguez et al., 2016). The block of inflammation-induced IR by the knockout of REG3β provides a new, effective strategy to neutralize the protein in preventing and treating the inflammation-related IR and diabetes that afflicts 10-20% the population (Spranger et al., 2003). Illustrating the relationship between their regulation of tissue REG3β and their impact on the inflammation-induced IR of five commonly used anti-inflammatory drugs not only helps explain the previously conflicting results in the clinic (Buren et al., 2002; da Silva et al., 2018; Ford et al., 2015), but also will guide the selection of these drugs in treating inflammation, in particular diabetic patients (Pollack et al., 2016).

4.2 Conclusion

In summary, our research reported an anti-inflammatory protein-REG3β is the key mediator of the inflammation-induced IR and its homologue in humans might be a novel target for ameliorating IR in type 2 diabetic patients clinically. Our research demonstrated REG3β inhibits the phosphorylation level of the insulin signaling pathway by binding to CXCL12/CXCR4 to activate SOCS3, and inhibits transcription of insulin

signaling pathway genes by binding to EXTL3 to activate the binding of NR3C1 with *Insr/Akt1/Akt2* promoter (Fig. 7). This discovery completes the traditional inflammation-IR model and provides a novel potential target to solve the inconsistency problem of AITs on IR and to treat similar human disorders of IR.

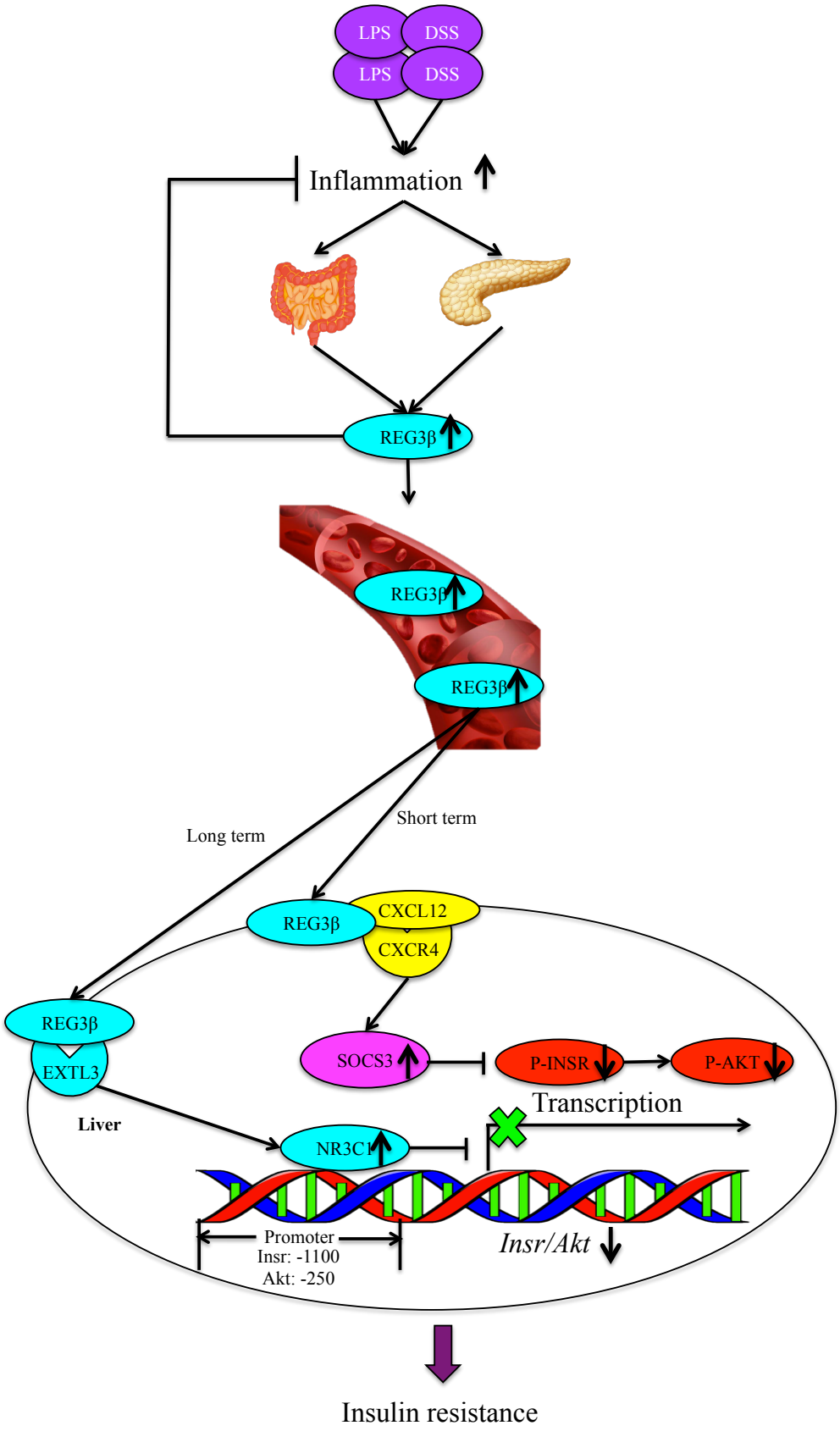
LPS
DSS
LPS
DSS
Inflammation
REG3β
REG3β
REG3β
Long term
Short term
REG3β
CXCL12
CXCR4
REG3β
EXTL3
SOCS3
P-INSR
P-AKT
Transcription
Liver
NR3C1
Promoter
Insr: -1100
Akt: -250
Insr/Akt
Insulin resistance

Figure 4.1 Postulated mechanistic scheme for impacts on insulin sensitivity mediated by the inflammation/REG3β/EXTL3/CXCR4/NR3C1/SOCS3 cascade in the liver. After inflammation induces the expression of REG3β in the pancreas and intestine, the secreted REG3β binds to transmembrane CXCR4 and its ligand CXCL12, leading to an acute inhibition of INSR and AKT phosphorylation. Meanwhile, the secreted REG3β binds to transmembrane EXTL3 and in turn stimulates the binding of NR3C1 to the proximate promoters of *Insr* and *Akt1* and *2*, resulting in transcriptional inhibition of these genes. In contrast, the lost binding of REG3β to EXTL3 and CXCR4 in the REG3β-deficient mice improves baseline insulin sensitivity and blocks the inflammation-induced insulin resistance.

TABLES

Table 1. Primers used for various purposes in this study

Gene Name	Forward (5'---3') Reverse (5'---3')
Primers for Q-PCR analysis of insulin signal, electron transportation chain and cell proliferation and apoptosis gene	
mInsr	GCCACTGTCATCAATGGGCAGTTT AGATAGAAGTTGCGACAGGCCACA
hInsr	TCCATTTCTGGGAATGAAGC GAGAGGAAACGCCACAAGAG
mAkt1	TATTGGCTACAAGGAACGGCCTCA TGTCTTCATCAGCTGGCATTGTGC
hAkt1	CATCACACCACCTGACCAAG CTCAAATGCACCCGAGAAAT
mAkt2	TAGCAGAATGCCAGCTGATG CTTGTAATCCATGGCGTCCT
hAkt2	GAGGTCATGGAGCACAGGTT CTGGCCGAGTAGGAGAACTG
mAkt3	AGGACCGCACACGTTTCTAT TCTGGTGTGCCACAGAATGT
hAkt3	GTTCGAGAGAAGGCAAGTGG TCGCCCCCATTAACATATTC
Irs1	CCCACAGCAGATCATTAACC AGAGACGAAGATGCTGGTGC
Irs2	GCCTGGGGATAATGGTGACTA TCCATGAGACTTAGCCGCTTC
Pi3k	AATAGGTTACAGTGCGGGCCGTAT CAGTTTCCTTGGCTTTGCTCGGTT
Pten	TGGATTACAGACCCGTGGCA ACAAACTGAGGATTGCAAGTTCC
hExtl3	TCACGCTCTTTGTCATCCTG GCTCTTCACTCACCGACTCC
Tnfα	CGTCAGCCGATTTGCTATCT TGGAAGACTCCTCCCAGGTA
Nfkb1	TTGGGAGAAGGCTGGAGAAG TGAACACAGGCTCATACGGT
P65	CACCGGATTGAAGAGAAGCG AGTTGAGTTTCGGGTAGGCA
Il1β	CCCAAGCAATACCCAAAGAA GCTTGTGCTCTGCTTGTGAG
Ikbkβ	TCAGTGCATCTCAGACAGCA TACAGCTGACACTTTCCGGT
Il6	AGACTTCCATCCAGTTGCCT CAGGTCTGTTGGGAGTGGTA
Il17	CAAACACTGAGGCCAAGGAC

Il22	GAGGTAGTCTGAGGGCCTTC TCCAACTTCCAGCAGCCATA TAGCACTGACTCCTCGGAAC
mβ-Actin	TCATGAAGTGTGACGTGGACATC CAGGAGGAGCAATGATCTTGATCT
hβ-Actin	GGACTTCGAGCAAGAGATGG AGCACTGTGTTGGCGTACAG

Primers for Q-PCR analysis of transcriptional factor genes with binding domains in the proximal promoter of Insr and Akt1-3 genes	
C/ebpd	ATCGACTTCAGCGCCTACAT CTAGCGACAGACCCCACAC
C/ebpβ	CAAGCTGAGCGACGAGTACA AGCTGCTCCACCTTCTTCTG
mHnf3b	GACATACCGACGCAGCTACA GGCACCTTGAGAAAGCAGTC
hHnf3b	CTACGCCAACATGAACTCCA CGGTAGAAGGGGAAGAGGTC
mNr3c1	AGGCCGCTCAGTGTTTTCTA TACAGCTTCCACACGTCAGC
hNr3c1	TACCCTGCATGTACGACCAA TCCTTCCCTCTTGACAATGG
Wt1	AGTTCCCCAACCATTCCTTC CATTCAAGCTGGGAGGTCAT

PCR primers used to create plasmid for luciferase assay[b]	
Insr P1 (-250/0)	AAAGGTACCAGCTAGTCCGTCGGTCCGC AAAGCTAGCGGCTCGATTTTGGCTTGG
Insr P2 (-500/0)	GGGGGTACCTCTGAAACTGGAGGAGAC AAAGCTAGCGGCTCGATTTTGGCTTGG
Insr P3 (-1000/0)	AAAGCTAGCGGTAGAGAAAGGATCTGT AAAGCTAGCGGCTCGATTTTGGCTTGG
Insr P4 (-1100/0)	AAAGGTACCTCAGAGATGTCCACCTGC AAAGCTAGCGGCTCGATTTTGGCTTGG
Akt1 P1 (-250/0)	GGGGGTACCTTTACAGACGGGGAAACT AAAGCTAGCCGCGGGGAGGAAATCCA
Akt1 P2 (-500/0)	AAAGGTACCCCCACCTCCAACTGACCT AAAGCTAGCCGCGGGGAGGAAATCCA
Akt1 P3 (-1000/0)	GGGGGTACCCTGAAAATAAAAATGCTC AAAGCTAGCCGCGGGGAGGAAATCCA
Akt2 P1 (-250/0)	GGGGGTACCTGAAAAGGAAACCCCTCC AAAGCTAGCAGACAACTTTGGGCCTGT
Akt2 P2 (-500/0)	GGGGGTACCCTGGAATATGTACGATGA AAAGCTAGCAGACAACTTTGGGCCTGT
Akt2 P3 (-1000/0)	AAAGGTACCCCCTATGAGAGAGGAGGG AAAGCTAGCAGACAACTTTGGGCCTGT

PCR primers used for ChIP assay[c]	
Insr P1 (-280/-1)	TTGGGTGTTTATGTTACACTGGT AGGGAAGGGACTGTGCG
Insr P2 (-520/-281)	CTCCAGACCTTTAAATTGGA CAACTCTAAGACTCACCTG
Insr P3 (-760/-521)	TGGTAGCATAGGCTGTAAAGA A CACACTCTAGATCTCCGCCA
Insr P4 (-1100/-761)	TGCTAGTGAATGGCTTTCTT AATGTCTTACGAGATTCTCC
Akt1 P1 (-760/-521)	TCGCCTATCCCCCAACCCAC CTCCTACTTCCCCCATCCCG
Akt1 P2 (-520/-281)	AGGAGGAGTTGGGGGCCTGG CAGCCACGCCCCATACCCTT
Akt1 P3 (-280/-1)	GAGAGGAGAGTGAGTTCTTCTCC GGCCAGCCTCAGCAGTTC
Akt1 P4 (-1000/-761)	GAAGAGGATATTCTAATGTGGG TCTGCCTTCTGAGCAATAGG
Akt2 P1 (-760/-521)	AGCAGGCAGATCTCTGAGTTC GCAGGCCAGAAGAGGGCATC
Akt2 P2 (-520/-281)	GCAGATCAGAGCACTCATACAT CTCACAGGGGTTGCCTAAGA
Akt2 P3 (-280/-1)	AGGTTCCCAAGTGAAGAACCGC GGCAGGCTCAAGGCAGCGCG
Akt2 P4 (-1000/-761)	GATCTCATTACGGGTGGTTG CTCCTTCTGGGTCCACACTT
Primers used to create exogenous Reg3ß/Cxcl12/Il-6	
pPICZαA-*Reg3ß*	CCGGAATTCGAAGACTCCCTGAAGAAT GCTCTAGAAAACCAGTAAATTTGCAGAC
pPICZαA-*Cxcl12*	AAAGAGCTCGACGCCAAGGTCGTC AAACTCGAGGTTTTTCCTTTTCTGGGCAG
pPICZαA-*Il-6*	AAAGAGCTCAAGTTCCTCTCTGCAAGA AAACTCGAGGGTTTGCCGAGTAGATCT
Primers used to create plasmid for AP-TAG binding and fluorescent-TAG binding assay	
AP-TAG *Reg3β*	GCTAGAATTCATGCTGCCTCCAACAG TTGTTCTAGACCACCAGTAAATTTGCA
pmCherry-*Extl3*	AAACTCGAGACAGGCTATACCATGTTG TGGAAGCTTCTAGATGAACTTGAAGCACT
pmCherry-*Cxcr4*	AAACTCGAGGAACCGATCAGTGTGAGT AAAGTCGACTTAGCTGAAGTGAAAACT
pcDNA-*Reg3-EGFP*	AAAAAGCTTATGCTGCCTCCAACACC GGGGGTACCACCAGTAAATTTGCAGAC

[a]Abbreviations: *Akt1*, AKT serine/threonine kinase 1; *Akt2*, AKT serine/threonine kinase 2; *Akt3*, AKT serine/threonine kinase 3; AP, alkaline phosphatase;*ß-Actin*, beta-actin; *C/ebpβ*, CCAAT/enhancer binding protein beta; *C/ebpd*, CCAAT/enhancer binding protein delta; *Cxcl12,* C-X-C motif chemokine ligand 12; *Cxcr4*, C-X-C motif chemokine receptor 4; EGFP, enhanced green fluorescent protein; *Extl3*, exostosin like glycosyltransferase 3; *Hnf3b*, Forkhead Box A2; *Ikbkβ,* inhibitor of nuclear factor kappa B kinase subunit beta; *Il1β,* interleukin 1β; *Il-6*, interleukin 6; *Il17,* interleukin 17; *Il22,* interleukin 22; *Insr*, insulin receptor; *Irs1*, insulin receptor subunit 1; *Irs2*, insulin receptor subunit 2; mCherry, mCherry fluorescent protein; *Nfkb1*, nuclear factor kappa B subunit 1; *Nr3c1*, glucocorticoid receptor; *P65*, RELA proto-oncogene, NF-KB subunit; *Pi3k*, phosphoinositide 3-kinase; *Pten*, phosphatase and tensin homolog; *Reg3β*, regenerating islet-derived *3β*; *Tnfα,* tumor necrosis factor-alpha; *Wt1*, wilms tumor 1

[b]PCR primers used to create plasmid for luciferase assays are named as amplification regions of each pair primer sets. -1, -280, -520, -760, -1000, -1100 = 1bp, 280bp, 520bp, 760bp, 1000bp, 1100 bp upstream of first nuclei acid in the first exon of *Insr/Akt1/Akt2*;

[c]PCR primers used for ChIP assays are named as the amplification regions of each pair primer sets.

Table 2. Primary and secondary antibodies used in this study

Antigen	Species	Working dilution	Buffer	Source
Goat-Anti-Rabbit	Goat	1:3000	3% milk	Bio-Rad Laboratories
Anti-Goat	Donkey	1:1000	3% milk	R&D Systems
Anti-Mouse	Goat	1:2000	3% milk	BD Pharmingen
Anti-Rat	Goat	1:1000	3% milk	R&D Systems
Donkey-Anti-Mouse	Donkey	1:100	3% BSA	Life technologies
ß-Actin[a]	Rabbit	1:1000	3% BSA	Cell Signaling Technology
P-Insr	Mouse	1:2000	3% BSA	Cell Signaling Technology
Insr	Rabbit	1:200	3% milk	Santa Cruz Biotechnology
P-Akt	Rabbit	1:2000	3% BSA	Cell Signaling Technology
Akt	Rabbit	1:1000	3% milk	Cell Signaling Technology
Socs3	Rabbit	1:1000	3% milk	IBL international
P-P38	Rabbit	1:1000	3% BSA	Cell Signaling Technology
P38	Rabbit	1:1000	3% milk	Cell Signaling Technology
P-Jnk	Rabbit	1:1000	3% BSA	Cell Signaling Technology
Jnk	Rabbit	1:1000	3% milk	Cell Signaling Technology
Cxcr4	Rabbit	1:5000	3% BSA	Novus Biologicals
His-tag	Mouse	1:1000	3% milk	Merck Millipore
Extl3	Mouse	1:100	3% milk	Santa Cruz Biotechnology
Reg3β	Rat	1:500	3% BSA	R&D Systems

[a]Abbreviations: *ß*-Actin, beta-actin; Akt, protein kinase B; Cxcr4, C-X-C motif chemokine receptor 4; Extl3, exostosin like glycosyltransferase 3; Gpx1, glutathione peroxidase 1; His-tag, polyhistidine-tag; Insr, insulin receptor; Jnk, the c-Jun NH2-terminal kinases; P-Akt, phosphor protein kinase B; P-Insr, phosphor insulin receptor; P-Jnk, phosphor c-Jun NH2-terminal kinases; P-P38, phosphor p38 mitogen-activated protein kinases; P38, p38 mitogen-activated protein kinases; Reg3β, regenerating islet-derived 3 beta; Socs3, suppressor of cytokine signaling 3;

Table 3. Plasmid used in this study

Plasmid[a]	Vector[b]	Inserted Gene	Host[c]	Vector Source
pPICZαA-*Reg3β*	pPICZαA	*Reg3β*	*P.pastoris* GS200	Invitrogen Corporation
pPICZαA-*Cxcl12*	pPICZαA	*Cxcl12*	*P.pastoris* GS200	
pPICZαA-*Il-6*	pPICZαA	*Il-6*	*P.pastoris* GS200	
pcDNA-EGFP	pcDNA 3.0	*Control*	HEK293T	
pcDNA-*Reg3β-EGFP*	pcDNA 3.0	*Reg3β*	HEK293T	
pmCherry-C3	pmCherry-C3	*Control*	HepG2	Addgene
pmCherry-C3-*Cxcr4*	pmCherry-C3	*Cxcr4*	HepG2	
pmCherry-C3-*Extl3*	pmCherry-C3	*Extl3*	HepG2	
pAPtag	pAPtag-5	*Control*	HEK293T	GenHunter Corporation
pAPtag-*Reg3β*	pAPtag-5	*Reg3β*	HEK293T	
pGL3	pGL3	*Control*	HepG2	
pGL3 (*Insr* -250/0)	pGL3	*Promoter of Insr* (-250/0)	HepG2	
pGL3 (*Insr* -500/0)	pGL3	*Promoter of Insr* (-500/0)	HepG2	
pGL3 (*Insr* -1100/0)	pGL3	*Promoter of Insr* (-1100/0)	HepG2	
pGL3 (*Akt1* -250/0)	pGL3	*Promoter of Akt1* (-250/0)	HepG2	
pGL3 (*Akt1* -500/0)	pGL3	*Promoter of Akt1* (-500/0)	HepG2	Promega Corporation
pGL3 (*Akt1* -1000/0)	pGL3	*Promoter of Akt1* (-1000/0)	HepG2	
pGL3 (*Akt2* -250/0)	pGL3	*Promoter of Akt2* (-250/0)	HepG2	
pGL3 (*Akt2* -500/0)	pGL3	*Promoter of Akt2* (-500/0)	HepG2	
pGL3 (*Akt2* -1000/0)	pGL3	*Promoter of Akt2* (-1000/0)	HepG2	
pRL-SV40	pRL-SV40	*Rl*	HepG2	

[a]Abbreviations: *Akt1*, AKT serine/threonine kinase 1; *Akt2*, AKT serine/threonine kinase 2; AP, alkaline phosphatase; *Cxcl12,* C-X-C motif chemokine ligand 12; *Cxcr4*, C-X-C motif chemokine receptor 4; EGFP, enhanced green fluorescent protein; *Extl3*, exostosin like glycosyltransferase 3; *Il-6*, interleukin 6; mCherry, mCherry fluorescent protein; *Reg3ß*, regenerating islet-derived *3ß*;

[b]pPICZαA,protein expression vector in yeast; pcDNA-EGFP 3.0, pmCherry-C3, pAPtag-5, pGL3, and pRL-SV40 protein expression vector in mammalian cell line.

[c]HepG2, human hepatocyte cell line; HEK293T, Human embryonic kidney 293t cell line.

CITATION

Aden, K., Rehman, A., Falk-Paulsen, M., Secher, T., Kuiper, J., Tran, F., Pfeuffer, S., Sheibani-Tezerji, R., Breuer, A., Luzius, A., et al. (2016). Epithelial IL-23R signaling licenses protective IL-22 responses in intestinal inflammation. Cell Rep *16*, 2208-2218.

Almlof, T., Wallberg, A.E., Gustafsson, J.A., and Wright, A.P.H. (1998). Role of important hydrophobic amino acids in the interaction between the glucocorticoid receptor tau 1-core activation domain and target factors. Biochemistry *37*, 9586-9594.

Boussif, O., Lezoualch, F., Zanta, M.A., Mergny, M.D., Scherman, D., Demeneix, B., and Behr, J.P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in-vivo - polyethylenimine. P Natl Acad Sci USA *92*, 7297-7301.

Buren, J., Liu, H.X., Jensen, J., and Eriksson, J.W. (2002). Dexamethasone impairs insulin signalling and glucose transport by depletion of insulin receptor substrate-1, phosphatidylinositol 3-kinase and protein kinase B in primary cultured rat adipocytes. Eur J endocrinol *146*, 419-429.

Busse, M., Feta, A., Presto, J., Wilen, M., Gronning, M., Kjellen, L., and Kusche-Gullberg, M. (2007). Contribution of EXT1, EXT2, and EXTL3 to heparan sulfate chain elongation. J Biol Chem *282*, 32802-32810.

Calderari, S., Irminger, J.C., Giroix, M.H., Ehses, J.A., Gangnerau, M.N., Coulaud, J., Rickenbach, K., Gauguier, D., Halban, P., Serradas, P., et al. (2014). Regenerating 1 and 3b gene expression in the pancreas of type 2 diabetic goto-kakizaki (GK) rats. PloS one *9*.

Carow, B., and Rottenberg, M.E. (2014). SOCS3, a major regulator of infection and inflammation. Front Immunol *5*, 58.

Chan, D.C., Watts, G.F., Barrett, P.H., Beilin, L.J., and Mori, T.A. (2002). Effect of atorvastatin and fish oil on plasma high-sensitivity C-reactive protein concentrations in individuals with visceral obesity. Clin Chem *48*, 877-883.

Chen, D.Y., Chen, Y.M., Hsieh, T.Y., Hsieh, C.W., Lin, C.C., and Lan, J.L. (2015). Significant effects of biologic therapy on lipid profiles and insulin resistance in patients with rheumatoid arthritis. Arthritis Res Ther *17*, 52.

Cheng, W.H., Zheng, X., Quimby, F.R., Roneker, C.A., and Lei, X.G. (2003). Low levels of glutathione peroxidase 1 activity in selenium-deficient mouse liver affect c-Jun N-terminal kinase activation and p53 phosphorylation on Ser-15 in pro-oxidant-induced aponecrosis. Biochem J *370*, 927-934.

da Silva, K.L.C., Camacho, A.P., Mittestainer, F.C., Carvalho, B.M., Santos, A., Guadagnini, D., Oliveira, A.G., and Saad, M.J.A. (2018). Atorvastatin and diacerein reduce insulin resistance and increase disease tolerance in rats with sepsis. J Inflamm *15*, 8.

Dahlmanwright, K., Almlof, T., Mcewan, I.J., Gustafsson, J.A., and Wright, A.P.H. (1994). Delineation of a small region within the major transactivation domain of the human glucocorticoid receptor that mediates transactivation of gene-expression. P Natl Acad Sci USA *91*, 1619-1623.

Duplan, L., Michel, B., Boucraut, J., Barthellemy, S., Desplat-Jego, S., Marin, V., Gambarelli, D., Bernard, D., Berthezene, P., Alescio-Lautier, B., et al. (2001). Lithostathine and pancreatitis-associated protein are involved in the very early stages of Alzheimer's disease. Neurobiol Aging *22*, 79-88.

Ebstein, W. (2002). Invited comment on W. Ebstein: On the therapy of diabetes mellitus, in particular on the application of sodium salicylate. J Mol Med *80*, 618; discussion 619.

Feve, B., and Bastard, J.P. (2009). The role of interleukins in insulin resistance and type 2 diabetes mellitus. Nature reviews. Endocrinology *5*, 305-311.

Finucane, O.M., Lyons, C.L., Murphy, A.M., Reynolds, C.M., Klinger, R., Healy, N.P., Cooke, A.A., Coll, R.C., McAllan, L., Nilaweera, K.N., et al. (2015). Monounsaturated fatty acid-enriched high-fat diets impede adipose NLRP3 inflammasome-mediated IL-1beta secretion and insulin resistance despite obesity. Diabetes *64*, 2116-2128.

Folch-Puy, E. (2013). REG3beta contributes to the immunosuppressive microenvironment of pancreatic cancer. Oncoimmunology *2*, e26404.

Folch-Puy, E., Granell, S., Dagorn, J.C., Iovanna, J.L., and Closa, D. (2006). Pancreatitis-associated protein I suppresses NF-kappa B activation through a JAK/STAT-mediated mechanism in epithelial cells. J Immunol *176*, 3774-3779.

Ford, R.J., Fullerton, M.D., Pinkosky, S.L., Day, E.A., Scott, J.W., Oakhill, J.S., Bujak, A.L., Smith, B.K., Crane, J.D., Blumer, R.M., et al. (2015). Metformin and salicylate synergistically activate liver AMPK, inhibit lipogenesis and improve insulin sensitivity. Biochemi J *468*, 125-132.

Froger, A., and Hall, J.E. (2007). Transformation of plasmid DNA into E. coli using the heat shock method. Journal of visualized experiments : JoVE, 253.

Fujii, W., Kohno, M., Ishino, H., Nakabayashi, A., Fujioka, K., Kida, T., Nagahara, H., Murakami, K., Nakamura, K., Seno, T., et al. (2012). The rapid efficacy of abatacept in a patient with rheumatoid vasculitis. Mod Rheumatol *22*, 630-634.

Galanternik, M.V., Kramer, K.L., and Piotrowski, T. (2015). Heparan sulfate proteoglycans regulate Fgf signaling and cell polarity during collective cell migration. Cell Rep *10*, 414-428.

Garcia Rodriguez, L.A., Martin-Perez, M., Hennekens, C.H., Rothwell, P.M., and Lanas, A. (2016). Bleeding risk with long-term low-dose aspirin: a systematic review of observational studies. PloS one *11*, e0160046.

Geer, E.B., Islam, J., and Buettner, C. (2014). Mechanisms of glucocorticoid-induced insulin resistance focus on adipose tissue function and lipid metabolism. Endocrin Metab Clin *43*, 75.

Germino, F.J., Wang, Z.X., and Weissman, S.M. (1993). Screening for in vivo protein-protein interactions. P Natl Acad Sci U S A *90*, 933-937.

Ghorpade, D.S., Ozcan, L., Zheng, Z., Nicoloro, S.M., Shen, Y., Chen, E., Bluher, M., Czech, M.P., and Tabas, I. (2018). Hepatocyte-secreted DPP4 in obesity promotes adipose inflammation and insulin resistance. Nature.

Gironella, M., Calvo, C., Fernandez, A., Closa, D., Iovanna, J.L., Rosello-Catafau, J., and Folch-Puy, E. (2013). Reg3beta deficiency impairs pancreatic tumor growth by skewing macrophage polarization. Cancer Res *73*, 5682-5694.

Gironella, M., Folch-Puy, E., LeGoffic, A., Garcia, S., Christa, L., Smith, A., Tebar, L., Hunt, S.P., Bayne, R., Smith, A.J., et al. (2007). Experimental acute pancreatitis in PAP/HIP knock-out mice. Gut *56*, 1091-1097.

Gironella, M., Iovanna, J.L., Sans, M., Gil, F., Penalva, M., Closa, D., Miquel, R., Pique, J.M., and Panes, J. (2005). Anti-inflammatory effects of pancreatitis associated protein in inflammatory bowel disease. Gut *54*, 1244-1253.

Hotamisligil, G.S., Shargill, N.S., and Spiegelman, B.M. (1993). Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science *259*, 87-91.

Iovanna, J.L., and Dagorn, J.C. (2005). The multifunctional family of secreted proteins containing a C-type lectin-like domain linked to a short N-terminal peptide. Biochim Biophys Acta *1723*, 8-18.

Jager, J., Gremeaux, T., Cormont, M., Le Marchand-Brustel, Y., and Tanti, J.F. (2007). Interleukin-1beta-induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression. Endocrinology *148*, 241-251.

Jorgensen, S.B., O'Neill, H.M., Sylow, L., Honeyman, J., Hewitt, K.A., Palanivel, R., Fullerton, M.D., Oberg, L., Balendran, A., Galic, S., et al. (2013). Deletion of skeletal muscle SOCS3 prevents insulin resistance in obesity. Diabetes *62*, 56-64.

Kang, S., Tsai, L.T., Zhou, Y., Evertts, A., Xu, S., Griffin, M.J., Issner, R., Whitton, H.J., Garcia, B.A., Epstein, C.B., et al. (2015). Identification of nuclear hormone receptor pathways causing insulin resistance by transcriptional and epigenomic analysis. Nat Cell Biol *17*, 44-56.

Keim, V., Iovanna, J.L., Rohr, G., Usadel, K.H., and Dagorn, J.C. (1991). Characterization of a rat pancreatic secretory protein associated with pancreatitis. Gastroenterology *100*, 775-782.

Kim, H.J., Higashimori, T., Park, S.Y., Choi, H., Dong, J., Kim, Y.J., Noh, H.L., Cho, Y.R., Cline, G., Kim, Y.B., et al. (2004). Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. Diabetes *53*, 1060-1067.

Lai, Y., Li, D., Li, C., Muehleisen, B., Radek, K.A., Park, H.J., Jiang, Z., Li, Z., Lei, H., Quan, Y., et al. (2012). The antimicrobial protein REG3A regulates keratinocyte proliferation and differentiation after skin injury. Immunity *37*, 74-84.

Larsen, C.M., Faulenbach, M., Vaag, A., Ehses, J.A., Donath, M.Y., and Mandrup-Poulsen, T. (2009). Sustained effects of interleukin-1 receptor antagonist treatment in type 2 diabetes. Diabetes Care *32*, 1663-1668.

Larsen, C.M., Faulenbach, M., Vaag, A., Volund, A., Ehses, J.A., Seifert, B., Mandrup-Poulsen, T., and Donath, M.Y. (2007). Interleukin-1-receptor antagonist in type 2 diabetes mellitus. N Engl J Med *356*, 1517-1526.

Li, P., Oh, D.Y., Bandyopadhyay, G., Lagakos, W.S., Talukdar, S., Osborn, O., Johnson, A., Chung, H., Maris, M., Ofrecio, J.M., et al. (2015). LTB4 promotes insulin resistance in obese mice by acting on macrophages, hepatocytes and myocytes. Nat Med *21*, 239-247.

Lin-Cereghino, J., Wong, W.W., Xiong, S., Giang, W., Luong, L.T., Vu, J., Johnson, S.D., and Lin-Cereghino, G.P. (2005). Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris. Biotechniques *38*, 44, 46, 48.

Lindemans, C.A., Calafiore, M., Mertelsmann, A.M., O'Connor, M.H., Dudakov, J.A., Jenq, R.R., Velardi, E., Young, L.F., Smith, O.M., Lawrence, G., et al. (2015). Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration. Nature *528*, 560-564.

Liu, J.L., Cui, W., Li, B., and Lu, Y. (2008). Possible roles of reg family proteins in pancreatic islet cell growth. Endocr Metab Immune Disord Drug Targets *8*, 1-10.

Liu, X., Wang, J., Wang, H., Yin, G., Liu, Y., Lei, X., and Xiang, M. (2015). REG3A accelerates pancreatic cancer cell growth under IL-6-associated inflammatory condition:

Involvement of a REG3A-JAK2/STAT3 positive feedback loop. Cancer Lett *362*, 45-60.

Loncle, C., Bonjoch, L., Folch-Puy, E., Lopez-Millan, M.B., Lac, S., Molejon, M.I., Chuluyan, E., Cordelier, P., Dubus, P., Lomberk, G., et al. (2015). IL-17 functions through the novel REG3beta-JAK2-STAT3 inflammatory pathway to promote the transition from chronic pancreatitis to pancreatic cancer. Cancer Res.

Lorchner, H., Poling, J., Gajawada, P., Hou, Y., Polyakova, V., Kostin, S., Adrian-Segarra, J.M., Boettger, T., Wietelmann, A., Warnecke, H., et al. (2015). Myocardial healing requires Reg3beta-dependent accumulation of macrophages in the ischemic heart. Nat Med *21*, 353-362.

Luo, C., Li, B., Liu, L., Yin, H.P., Wang, M., and Liu, J.L. (2013). Transcriptional activation of Reg2 and Reg3beta genes by glucocorticoids and interleukin-6 in pancreatic acinar and islet cells. Mol Cell Endocrinol *365*, 187-196.

Macdougall, C.E., Wood, E.G., Loschko, J., Scagliotti, V., Cassidy, F.C., Robinson, M.E., Feldhahn, N., Castellano, L., Voisin, M.B., Marelli-Berg, F., et al. (2018). Visceral adipose tissue immune homeostasis is regulated by the crosstalk between adipocytes and dendritic cell subsets. Cell Metab *27*, 588-601 e584.

Makrilakis, K., Fragiadaki, K., Smith, J., Sfikakis, P.P., and Kitas, G.D. (2015). Interrelated reduction of chemerin and plasminogen activator inhibitor-1 serum levels in rheumatoid arthritis after interleukin-6 receptor blockade. Clin Rheumatol *34*, 419-427.

Matsubara, T., Mita, A., Minami, K., Hosooka, T., Kitazawa, S., Takahashi, K., Tamori, Y., Yokoi, N., Watanabe, M., Matsuo, E., et al. (2012). PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue. Cell Metab *15*, 38-50.

Matsumoto, M., Han, S., Kitamura, T., and Accili, D. (2006). Dual role of transcription factor FoxO1 in controlling hepatic insulin sensitivity and lipid metabolism. J Clin Invest *116*, 2464-2472.

McClung, J.P., Roneker, C.A., Mu, W., Lisk, D.J., Langlais, P., Liu, F., and Lei, X.G. (2004). Development of insulin resistance and obesity in mice overexpressing cellular glutathione peroxidase. P Natl Acad Sci U S A *101*, 8852-8857.

Merrill, J.T., Burgos-Vargas, R., Westhovens, R., Chalmers, A., D'Cruz, D., Wallace, D.J., Bae, S.C., Sigal, L., Becker, J.C., Kelly, S., et al. (2010). The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. Arthritis Rheum *62*, 3077-3087.

Miki, T., Holst, O., and Hardt, W.D. (2012). The bactericidal activity of the C-type lectin RegIIIbeta against Gram-negative bacteria involves binding to lipid A. J Biol Chem *287*, 34844-34855.

Mukherjee, S., Zheng, H., Derebe, M.G., Callenberg, K.M., Partch, C.L., Rollins, D., Propheter, D.C., Rizo, J., Grabe, M., Jiang, Q.X., et al. (2014). Antibacterial membrane attack by a pore-forming intestinal C-type lectin. Nature *505*, 103-107.

Nakai, J., Biggs, W.H., Kitamura, T., Cavenee, W.K., Wright, C.V.E., Arden, K.C., and Accili, D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nature Genet *32*, 245-253.

Nieto-Vazquez, I., Fernandez-Veledo, S., de Alvaro, C., and Lorenzo, M. (2008). Dual role of interleukin-6 in regulating insulin sensitivity in murine skeletal muscle. Diabetes *57*, 3211-3221.

Ogawa, H., Fukushima, K., Naito, H., Funayama, Y., Unno, M., Takahashi, K., Kitayama, T., Matsuno, S., Ohtani, H., Takasawa, S., et al. (2003). Increased expression of HIP/PAP and regenerating gene III in human inflammatory bowel disease and a murine bacterial reconstitution model. Inflamm Bowel Dis *9*, 162-170.

Orelle, B., Keim, V., Masciotra, L., Dagorn, J.C., and Iovanna, J.L. (1992). Human pancreatitis-associated protein. Messenger RNA cloning and expression in pancreatic diseases. J Clin Invest *90*, 2284-2291.

Pereira, M.J., Palming, J., Svensson, M.K., Rizell, M., Dalenback, J., Hammar, M., Fall, T., Sidibeh, C.O., Svensson, P.A., and Eriksson, J.W. (2014). FKBP5 expression in human adipose tissue increases following dexamethasone exposure and is associated with insulin resistance. Metab Clin Exp *63*, 1198-1208.

Perry, C.G., Spiers, A., Cleland, S.J., Lowe, G.D., Petrie, J.R., and Connell, J.M. (2003). Glucocorticoids and insulin sensitivity: dissociation of insulin's metabolic and vascular actions. J Clin Endocrinol Metab *88*, 6008-6014.

Perry, R.J., Camporez, J.P., Kursawe, R., Titchenell, P.M., Zhang, D., Perry, C.J., Jurczak, M.J., Abudukadier, A., Han, M.S., Zhang, X.M., et al. (2015). Hepatic acetyl CoA links adipose tissue inflammation to hepatic insulin resistance and type 2 diabetes. Cell *160*, 745-758.

Pickert, G., Neufert, C., Leppkes, M., Zheng, Y., Wittkopf, N., Warntjen, M., Lehr, H.A., Hirth, S., Weigmann, B., Wirtz, S., et al. (2009). STAT3 links IL-22 signaling in intestinal epithelial cells to mucosal wound healing. J Exp Med *206*, 1465-1472.

Pickup, J.C., Mattock, M.B., Chusney, G.D., and Burt, D. (1997). NIDDM as a disease of the innate immune system: association of acute-phase reactants and interleukin-6 with metabolic syndrome X. Diabetologia *40*, 1286-1292.

Pollack, R.M., Donath, M.Y., LeRoith, D., and Leibowitz, G. (2016). Anti-inflammatory agents in the treatment of diabetes and its vascular complications. Diabetes Care *39 Suppl 2*, S244-252.

Preiss, D., Seshasai, S.R., Welsh, P., Murphy, S.A., Ho, J.E., Waters, D.D., DeMicco, D.A., Barter, P., Cannon, C.P., Sabatine, M.S., et al. (2011). Risk of incident diabetes with intensive-dose compared with moderate-dose statin therapy: a meta-analysis. Jama *305*, 2556-2564.

Puig, O., and Tjian, R. (2005). Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes Dev *19*, 2435-2446.

Ramadoss, P., Unger-Smith, N.E., Lam, F.S., and Hollenberg, A.N. (2009). STAT3 targets the regulatory regions of gluconeogenic genes in vivo. Mol Endocrinol *23*, 827-837.

Rankin, M.M., and Kushner, J.A. (2010). Aging induces a distinct gene expression program in mouse islets. Islets *2*, 345-352.

Reid, J., Macdougall, A.I., and Andrews, M.M. (1957). Aspirin and diabetes mellitus. BMJ *2*, 1071-1074.

Schug, J. (2008). Using TESS to predict transcription factor binding sites in DNA sequence. Curr Protoc Bioinformatics *Chapter 2*, Unit 2.6.

Severgnini, M., Sherman, J., Sehgal, A., Jayaprakash, N.K., Aubin, J., Wang, G., Zhang, L., Peng, C.G., Yucius, K., Butler, J., et al. (2012). A rapid two-step method for isolation of functional primary mouse hepatocytes: cell characterization and asialoglycoprotein receptor based assay development. Cytotechnology *64*, 187-195.

Spranger, J., Kroke, A., Mohlig, M., Hoffmann, K., Bergmann, M.M., Ristow, M., Boeing, H., and Pfeiffer, A.F. (2003). Inflammatory cytokines and the risk to develop type 2 diabetes: results of the prospective population-based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. Diabetes *52*, 812-817.

Sund, N.J., Vatamaniuk, M.Z., Casey, M., Ang, S.L., Magnuson, M.A., Stoffers, D.A., Matschinsky, F.M., and Kaestner, K.H. (2001). Tissue-specific deletion of Foxa2 in pancreatic beta cells results in hyperinsulinemic hypoglycemia. Genes Dev *15*, 1706-1715.

Takekoshi, T., Wu, X., Mitsui, H., Tada, Y., Kao, M.C., Sato, S., Dwinell, M.B., and Hwang, S.T. (2013). CXCR4 negatively regulates keratinocyte proliferation in IL-23-

mediated psoriasiform dermatitis. J Invest Dermatol *133*, 2530-2537.

Thompson, J.D., Gibson, T.J., and Higgins, D.G. (2002). Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics *Chapter 2*, Unit 2 3.

Tian, X.Y., Ganeshan, K., Hong, C., Nguyen, K.D., Qiu, Y., Kim, J., Tangirala, R.K., Tontonoz, P., and Chawla, A. (2016). Thermoneutral housing accelerates metabolic inflammation to potentiate atherosclerosis but not insulin resistance. Cell Metab *23*, 165-178.

Tournadre, A., Pereira, B., Dutheil, F., Giraud, C., Courteix, D., Sapin, V., Frayssac, T., Mathieu, S., Malochet-Guinamand, S., and Soubrier, M. (2017). Changes in body composition and metabolic profile during interleukin 6 inhibition in rheumatoid arthritis. J Cachexia Sarcopenia Muscle *8*, 639-646.

Truong, K., and Ikura, M. (2001). The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. Curr Opin Struc Biol *11*, 573-578.

van Ampting, M.T.J., Loonen, L.M.P., Schonewille, A.J., Konings, I., Vink, C., Iovanna, J., Chamaillard, M., Dekker, J., van der Meer, R., Wells, J.M., et al. (2012). Intestinally secreted c-type lectin Reg3b attenuates salmonellosis but not listeriosis in mice. Infect Immun *80*, 1115-1120.

van Asseldonk, E.J., van Poppel, P.C., Ballak, D.B., Stienstra, R., Netea, M.G., and Tack, C.J. (2015). One week treatment with the IL-1 receptor antagonist anakinra leads to a sustained improvement in insulin sensitivity in insulin resistant patients with type 1 diabetes mellitus. Clin Immunol *160*, 155-162.

Van Ba, I.A.T., Marchal, S., Francois, F., Silhol, M., Lleres, C., Michel, B., Benyamin, Y., Verdier, J.M., Trousse, F., and Marcilhac, A. (2012). Regenerating Islet-derived 1 alpha (Reg-1 alpha) protein is new neuronal secreted factor that stimulates neurite outgrowth via exostosin tumor-like 3 (EXTL3) receptor. J Biol Chem *287*, 4726-4739.

van Raalte, D.H., van Leeuwen, N., Simonis-Bik, A.M., Nijpels, G., van Haeften, T.W., Schafer, S.A., Boomsma, D.I., Kramer, M.H.H., Heine, R.J., Maassen, J.A., et al. (2012). Glucocorticoid receptor gene polymorphisms are associated with reduced first-phase glucose-stimulated insulin secretion and disposition index in women, but not in men. Diabetic Med *29*, E211-E216.

van Rossum, E.F.C., Koper, J.W., Huizenga, N.A.T.M., Uitterlinden, A.G., Janssen, J.A.M.J.L., Brinkmann, A.O., Grobbee, D.E., de Jong, F.H., van Duyn, C.M., Pols, H.A.P., et al. (2002). A polyntorphism in the glucocorticoid receptor gene, which decreases sensitivity to Glucocorticoids in vivo, is associated with low insulin and cholesterol levels. Diabetes *51*, 3128-3134.

Ventre, J., Doebber, T., Wu, M., MacNaul, K., Stevens, K., Pasparakis, M., Kollias, G., and Moller, D.E. (1997). Targeted disruption of the tumor necrosis factor-alpha gene: metabolic consequences in obese and nonobese mice. Diabetes *46*, 1526-1531.

Wang, J., Harada, A., Matsushita, S., Matsumi, S., Zhang, Y., Shioda, T., Nagai, Y., and Matsushima, K. (1998). IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4+ T lymphocytes and enhance HIV-1 replication. J Leukoc Biol *64*, 642-649.

Wang, J.C., Derynck, M.K., Nonaka, D.F., Khodabakhsh, D.B., Haqq, C., and Yamamoto, K.R. (2004). Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. P Natl Acad Sci U S A *101*, 15603-15608.

Wang, X.D., Vatamaniuk, M.Z., Wang, S.K., Roneker, C.A., Simmons, R.A., and Lei, X.G. (2008). Molecular mechanisms for hyperinsulinaemia induced by overproduction of selenium-dependent glutathione peroxidase-1 in mice. Diabetologia *51*, 1515-1524.

Wascher, T.C., Lindeman, J.H.N., Sourij, H., Kooistra, T., Pacini, G., and Roden, M. (2011). Chronic TNF-alpha neutralization does not improve insulin resistance or endothelial function in "healthy" men with metabolic syndrome. Mol Med *17*, 189-193.

Wensveen, F.M., Jelencic, V., Valentic, S., Sestan, M., Wensveen, T.T., Theurich, S., Glasner, A., Mendrila, D., Stimac, D., Wunderlich, F.T., et al. (2015). NK cells link obesity-induced adipose stress to inflammation and insulin resistance. Nature Immunol *16*, 376-385.

Wescott, M.P., Kufareva, I., Paes, C., Goodman, J.R., Thaker, Y., Puffer, B.A., Berdougo, E., Rucker, J.B., Handel, T.M., and Doranz, B.J. (2016). Signal transmission through the CXC chemokine receptor 4 (CXCR4) transmembrane helices. P Natl Acad Sci U S A *113*, 9928-9933.

Wu, S., Dong, K., Wang, J., and Bi, Y. (2018). Tumor necrosis factor alpha improves glucose homeostasis in diabetic mice independent with tumor necrosis factor receptor 1 and tumor necrosis factor receptor 2. Endocr J.

Wu, Y., Quan, Y., Liu, Y., Liu, K., Li, H., Jiang, Z., Zhang, T., Lei, H., Radek, K.A., Li, D., et al. (2016a). Hyperglycaemia inhibits REG3A expression to exacerbate TLR3-mediated skin inflammation in diabetes. Nat Commun *7*, 13393.

Wu, Y.L., Quan, Y.C., Liu, Y.Q., Liu, K.W., Li, H.Q., Jiang, Z.W., Zhang, T., Lei, H., Radek, K.A., Li, D.Q., et al. (2016b). Hyperglycaemia inhibits REG3A expression to exacerbate TLR3-mediated skin inflammation in diabetes. Nat Commun *7*.

Wynn, T.A., and Vannella, K.M. (2016). Macrophages in tissue repair, regeneration, and fibrosis. Immunity *44*, 450-462.

Xiang, M., Wang, P.X., Wang, A.B., Zhang, X.J., Zhang, Y., Zhang, P., Mei, F.H., Chen, M.H., and Li, H. (2016). Targeting hepatic TRAF1-ASK1 signaling to improve inflammation, insulin resistance, and hepatic steatosis. J Hepatol *64*, 1365-1377.

Xie, M.J., Motoo, Y., Iovanna, J.L., Su, S.B., Ohtsubo, K., Matsubara, F., and Sawabu, N. (2003). Overexpression of pancreatitis-associated protein (PAP) in human pancreatic ductal adenocarcinoma. Dig Dis Sci *48*, 459-464.

Xiong, X., Li, Q., Cui, W., Gao, Z.H., and Liu, J.L. (2016). Deteriorated high-fat diet-induced diabetes caused by pancreatic beta-cell-specific overexpression of Reg3beta gene in mice. Endocrine *54*, 360-370.

Yamada, E., Hirota, T., Hatori, N., Kitao, Y., Fuse, Y., Aoki, S., Karatani, H., and Matsunaga, T. (2012). Characterization of protein-like fluorophores released from lake phytoplankton on the basis of fractionation and electrophoresis. Anal Sci *28*, 595-600.

Yan, X., Pepper, M.P., Vatamaniuk, M.Z., Roneker, C.A., Li, L., and Lei, X.G. (2012). Dietary selenium deficiency partially rescues type 2 diabetes-like phenotypes of glutathione peroxidase-1-overexpressing male mice. J Nutr *142*, 1975-1982.

Yun, J.W., Lum, K., and Lei, X.G. (2013). A novel upregulation of glutathione peroxidase 1 by knockout of liver-regenerating protein Reg3beta aggravates acetaminophen-induced hepatic protein nitration. Free Radic Biol Med *65*, 291-300.

Zhang, L., Badgwell, D.B., Bevers, J.J., Schlessinger, K., Murray, P.J., Levy, D.E., and Watowich, S.S. (2006). IL-6 signaling via the STAT3/SOCS3 pathway: Functional analysis of the conserved STAT3 N-domain. Mol Cell Biochem *288*, 179-189.

Zhang, L., Li, J., Li, L., Zhang, J., Wang, X.D., Yang, C.H., Li, Y.Y., Lan, F., and Lin, P. (2014). IL-23 selectively promotes the metastasis of colorectal carcinoma cells with impaired Socs3 expression via the STAT5 pathway. Carcinogenesis *35*, 1330-1340.

Zhong, B., Strnad, P., Toivola, D.M., Tao, G.Z., Ji, X., Greenberg, H.B., and Omary, M.B. (2007). Reg-II is an exocrine pancreas injury-response product that is up-regulated by keratin absence or mutation. Mol Biol Cell *18*, 4969-4978.